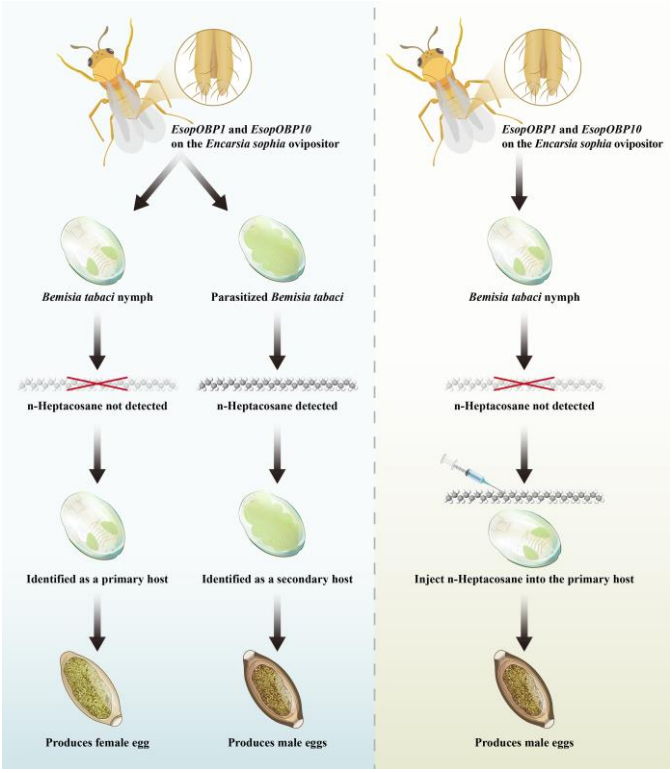
LIÈGE université
Gembloux
Agro-Bio Tech

中国农业科学院
CHINESE ACADEMY OF AGRICULTURAL SCIENCES

The host adaptive mechanism of the heteronomous parasitoid *Encarsia sophia*



Xiaoming Man

Promoters : **Prof. Frédéric Francis**

Prof. Wanxue Liu

Prof. Nianwan Yang

2024

FRENCH COMMUNITY OF BELGIUM
UNIVERSITY OF LIÈGE – GEMBLOUX AGRO-BIO TECH
BELGIUM

Host adaptive mechanism of the heteronomous parasitoid *Encarsia sophia*

Xiaoming Man

Original assay for graduation as a doctor in agricultural sciences and biological engineering

Promoters : Prof. Frédéric Francis
Prof. Wanxue Liu
Prof. Nianwan Yang

Civil year : 2024

Abstract

This study investigated the reproductive adaptive strategies of the heteronomous hyperparasitoid *Encarsia sophia*, a dominant parasitoid of the *Bemisia tabaci* "super pest". Through behavioral, physiological, molecular, transcriptomic, and genomic approaches, key regulatory mechanisms underlying its parasitic behavior were revealed.

In Chapter 3, the ability of *E. sophia* to adjust its offspring sex ratio under varying host resource conditions was examined. It was demonstrated that *E. sophia* can modify the sex ratio in favor of female offspring when host density is low or the proportion of secondary hosts is high, thereby improving biological control efficiency. Optimal rearing conditions, with a secondary host proportion of 0.2 and a host density of 3/ cm^2, were identified as a reference for large-scale production.

The chromosome-level genome assembly of *E. sophia* was reported in Chapter 4 to be assembled into five chromosomes using Hi-C technology with a mapping rate of 95.13%. A total of 14,914 protein-coding genes were predicted, representing the first complete genome of a heteronomous hyperparasitoid. This provides a crucial genomic foundation for further exploration of its evolutionary mechanisms and host interactions.

In Chapter 5, the molecular mechanism by which *E. sophia* females decide whether to lay male or female eggs based on host odor cues was studied. Olfactory receptors on the ovipositor were identified, and n-heptacosane was found to be a secondary host-specific volatile that induced the laying of male eggs. Two key odorant-binding proteins (*EsopOBP1* and *EsopOBP10*) were further identified, highlighting their essential role in oviposition decision-making.

Overall, this research systematically elucidates the mechanisms of sex allocation regulation, genomic characteristics and the molecular basis of oviposition decisions in *E. sophia*, providing important theoretical insights for optimizing the large-scale application of heteronomous hyperparasitoids in biological control.

Keywords: *Encarsia sophia,* heteronomous hyperparasitism, sex allocation, genome, oviposition mechanism

Résumé

Cette étude a investigué les stratégies adaptatives de reproduction de l'hyperparasitoïde hétéronome *Encarsia sophia*, un parasitoïde dominant du "super ravageur" *Bemisia tabaci*. À travers des approches comportementales, physiologiques, moléculaires, transcriptomiques et génomiques, les mécanismes de régulation clés sous-jacents à son comportement parasitaire ont été révélés.

Dans le chapitre 3, la capacité de *E. sophia* à ajuster le sexe ratio de sa progéniture en fonction des conditions de ressources des hôtes a été examinée. Il a été démontré que *E. sophia* peut modifier le sexe ratio en faveur des femelles lorsque la densité des hôtes est faible ou que la proportion d'hôtes secondaires est élevée, améliorant ainsi l'efficacité de la lutte biologique. Les conditions optimales d'élevage, avec une proportion d'hôtes secondaires de 0,2 et une densité d'hôtes de 3/ cm^2, ont été identifiées comme référence pour la production à grande échelle.

Le chapitre 4 rapporte l'assemblage du génome à l'échelle chromosomique de *E. sophia*, organisé en cinq chromosomes à l'aide de la technologie Hi-C, avec un taux de cartographie de 95,13%. Un total de 14 914 gènes codant des protéines ont été prédits, représentant le premier génome complet d'un hyperparasitoïde hétéronome. Cela fournit une base génomique essentielle pour explorer plus avant ses mécanismes évolutifs et ses interactions avec les hôtes.

Dans le chapitre 5, le mécanisme moléculaire par lequel les femelles d' *E. sophia* décident de produire des œufs mâles ou femelles en fonction des signaux olfactifs des hôtes a été étudié. Des récepteurs olfactifs ont été identifiés sur l'ovipositeur, et il a été découvert que le n-heptacosane est un composé volatil spécifique aux hôtes secondaires, induisant le dépôt d'œufs mâles. Deux protéines de liaison aux odeurs clés (*EsopOBP1* et *EsopOBP10*) ont été identifiées, soulignant leur rôle essentiel dans la prise de décision de ponte.

Dans l'ensemble, cette recherche élucide systématiquement les mécanismes de régulation du sexe ratio, les caractéristiques génomiques et la base moléculaire des décisions de ponte chez *E. sophia*, fournissant des perspectives théoriques importantes pour optimiser l'application à grande échelle des hyperparasitoïdes hétéronomes dans la lutte biologique.

Mots-clés: *Encarsia sophia*, hyperparasitisme hétéronome, allocation des sexes, génome, mécanisme de ponte

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my promoters, Professor **Frédéric Francis** (Functional & Evolutionary Entomology, University of Liege – Gembloux Agro-Bio Tech, Belgium) and Professor **Wanxue Liu** (Institute of Plant Protection, Chinese Academy of Agricultural Sciences, China) for their patient guidance, erudite knowledge and valuable suggestions from the beginning to the end of this Ph.D. project.

I gratefully acknowledge the financial scholarship from the National Natural Science Foundation of China (32072493) and China Scholarship Council (202203250080), the support associated with studying and researching facilities from **University of Liege – Gembloux Agro-Bio Tech** (Belgium) and **Chinese Academy of Agricultural Sciences** (China), the assistance related to data collection, project communication, and research guidance from Professor **Nianwan Yang** (Institute of Plant Protection, Chinese Academy of Agricultural Sciences, China). Thanks to Professor **Mingjun Zhang** (Graduate School of Chinese Academy of Agricultural Sciences, China) for his invaluable assistance and unwavering support throughout the course of my doctoral studies. Sincere thanks to **all members of my thesis committee** for their meticulous guidance, which has been of great assistance to me. Undoubtably, their support has been instrumental in shaping the course of my academic journey.

I wish to acknowledge that, beyond my individual endeavors, the accomplishment of this thesis is significantly indebted to the encouragement and guidance extended by numerous individuals. I seize this moment to convey my appreciation to all those who have generously contributed their assistance and offered invaluable counsel during this undertaking, encompassing, though not confined to, **my friends and colleagues**.

Last but not least, I would like to wholeheartedly express my profound gratitude to my cherished family. Their unwavering belief in my abilities, understanding, and the immeasurable sacrifices they have made alongside me have always been my rock and inspiration.

Table of contents

List of figures

List of tables

List of acronyms

BB: Böhm Bristles

BLAST: Basic Local Alignment Search Tool

BUSCO: Benchmarking Universal Single-Copy Orthologs

BWA: Burrows-Wheeler Aligner

CDS: Coding Sequence

CEGMA: Core Eukaryotic Genes Mapping Approach

DS: Dentate Sensilla

DV: Dorsal Valves

EVM: EVidenceModeler

GC-MS: Gas Chromatography-Mass Spectrometry

GLVs: Green Leaf Volatiles

GO: Gene Ontology

Hi-C: Chromosome Conformation Capture

HIPVs: Herbivore-Induced Plant Volatiles

KEGG: Kyoto Encyclopedia of Genes and Genomes

LINE: Long Interspersed Nuclear Element

LMC: Local Mate Competition

LTR: Long Terminal Repeat

MAFFT: Multiple Alignment using Fast Fourier Transform

MCMC: Markov Chain Monte Carlo

MVT: Marginal Value Theorem

mya: Million Years Ago

NR: Non-Redundant Protein

OR: Odorant Receptor

OS: Ovipositor Sheath

PacBio: Pacific Biosciences

PAML: Phylogenetic Analysis by Maximum Likelihood

PASA: Program to Assemble Spliced Alignments

RAxML: Randomized Accelerated Maximum Likelihood

RNA-seq: RNA Sequencing

rRNA: Ribosomal RNA

SB: Sensilla Basiconica

SC: Sensilla Coeloclnica

SCa: Sensilla Campaniformia

SD: Slight surface Depression

SINE: Short Interspersed Nuclear Element

SMRT: Single Molecule, Real-Time

SNAP: SNP Annotation and Proxy Search

snRNA: Small Nuclear RNA

TRF: Tandem Repeats Finder; tRNA: Transfer RNA

VOCs: Volatile Organic Compounds

VV: Ventral Valves

WM-FISH: Whole Mount Fluorescence In Situ Hybridization

Chapter 1

The adaptive strategies of reproductive fitness in parasitoids wasps: a review

Abstract

Parasitoid wasps are a group of insects with significant ecological and economic value, exhibiting highly adaptive and diverse reproductive behaviors and strategies in natural environments. This review provides an overview of the various reproductive strategies of parasitoid wasps, including mating, oviposition, host defense mechanisms, and nutrient acquisition, and explores how these strategies maximize reproductive success in changing environments. The review highlights how parasitoid wasps enhance reproductive success through strategies such as multiple mating, inbreeding avoidance, and sexual selection. It also discusses how host quality assessment, competitive strategies, and patch time allocation optimize offspring survival. Additionally, parasitoid wasps have evolved immune evasion and nutrient utilization strategies to maximize reproductive potential under limited resource conditions. Future research should systematically explore the diverse adaptive strategies and evolutionary mechanisms of parasitoid wasps within complex ecosystems, particularly in the context of behavioral adaptation and physiological regulation under climate change, to enhance their application in natural pest control.

***Keywords*:** Parasitoid wasps, reproductive fitness, mating, oviposition, immune suppression, nutrient utilization

1. Introduction

Reproduction is one of the most fundamental behavioral activities of insects and a crucial means for ensuring the continuation and prosperity of their populations. As an indicator for assessing the strength and changes in insect reproductive capacity, the study of insect reproductive fitness is a population-level concept that refers to the ability of insects to produce offspring in a natural environment that can adapt to their surroundings. This includes the survival ability of the parents, their reproductive capacity and the survival ability of the offspring (Roy et al., 2018). Parasitoid wasps are a type of insect belonging to the order Hymenoptera, with a lifestyle that falls between parasitism and predation. Most parasitoids are wasps whose adult forms typically feed on nectar or other plant fluids and lay their eggs on or inside the early developmental stages (eggs, larvae or pupae) of other insects. When the early stages of endoparasitoids escape or overcome the host's immune defenses, their larvae develop by feeding on the nutrients of the host insect or arthropod, either within or on the host's body (Godfray, 1994; Wang et al., 2019). The larvae consume the host's body fluids and tissues until the host dies, after which they pupate and emerge as free-living adults. Because successful parasitism inevitably results in the host's death, parasitoids are a significant (and possibly the primary) regulatory factor of insect populations. They play a vital role in regulating agricultural pest populations, maintaining ecological balance, and preserving biodiversity within ecosystems (Burke, 2024). The reproductive fitness of parasitoids refers to their ability to successfully complete their life cycle through parasitic behavior and effectively pass their genes to the next generation under specific environmental conditions. This concept encompasses various aspects: reproductive success is primarily measured by the mating success rate of adults, oviposition behavior, the defensive strategies of larvae against the host and the efficiency of nutrient utilization for their survival and reproduction (Stearns, 1992; Shuker and West, 2004; Harvey, 2005). The reproductive fitness of parasitoids reflects their adaptability and competitiveness within the ecosystem and is a crucial indicator for assessing population dynamics and the potential for biological control (Quicke, 1997; Lucie et al., 2021; Alena et al., 2022). The evolution of life history traits has led to complex adaptations that maximize fitness in local environments (Stearns, 1992; Ye et al., 2024). Under natural conditions, parasitoids have only limited resources available for the development of their offspring and must also cope with intra- and interspecific competition, variations in host quality and density, food shortages and the host's defenses. In response to these challenges, each parasitoid wasp employs certain reproductive strategies to maximize its fitness. Over time, natural selection leads to adaptations to local environmental conditions (Grillenberger, 2009). We will summarize the reproductive strategies that parasitoids adopt to enhance their fitness by examining their behaviors throughout their life, including mating, oviposition, host defenses, and feeding (Figure 1-1) and explore how these strategies promote their survival and reproduction under different environmental conditions. By gaining a deeper understanding of these strategies, we can not only reveal how parasitoids overcome survival challenges during evolution

but also clarify their crucial role in ecosystems, particularly their value in the biological control of natural pests.

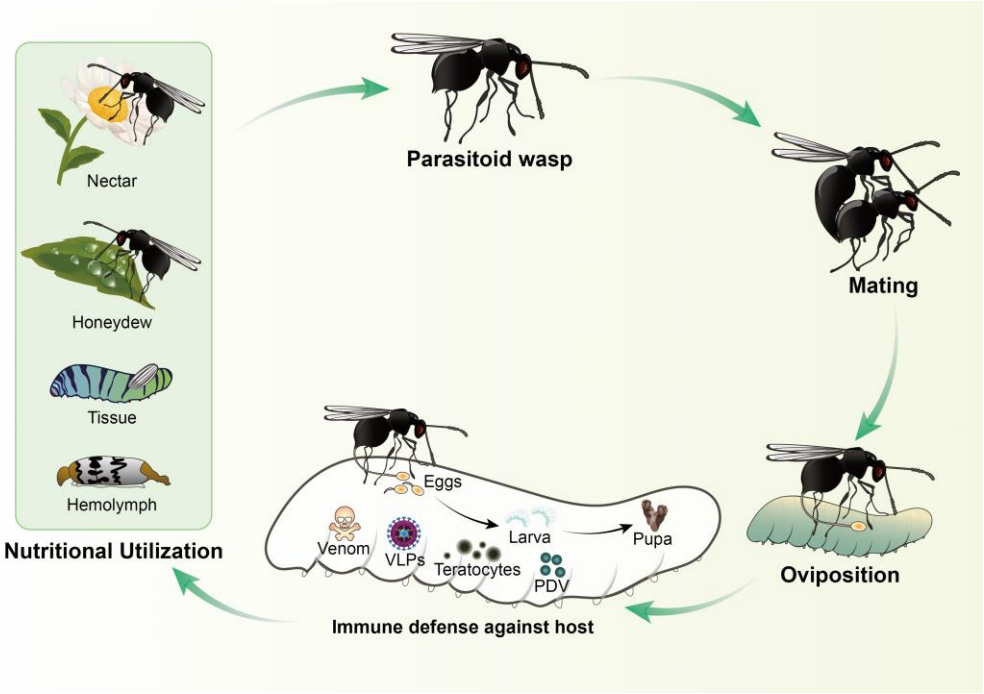


Figure 1-1. Reproductive strategies based on parasitoid wasp mating, oviposition, host defense, and nutrient utilization.

2. The lifestyle and reproductive behavioral diversity of parasitoid wasps

The reproductive behavior of parasitoids refers to a series of actions or activities involved in the continuation of their species (Qin, 2009). This behavior is diverse in its forms and can be classified based on whether the eggs are fertilized or not. It includes asexual reproduction and sexual reproduction, with the former further divided into arrhenotoky (unfertilized eggs develop into males) and thelytoky (unfertilized eggs develop into females) (Kuo and Kang, 2024). Parasitoids exhibit a typical haplodiploid sex determination system, where unfertilized haploid eggs develop into males and fertilized diploid eggs develop into females (West et al., 2003). The diversity of reproductive behavior is closely associated with the species and habits of parasitoids (Polaszek and Vilhemsen, 2023) (Table 1-1). Parasitoids are further classified based on their effects on the host's physiological state. They can be categorized into idiobiont parasitoids, where the female injects toxins into the host during oviposition, causing paralysis or arresting host development until death, and koinobiont parasitoids, where the host continues to develop and mount immune responses after parasitization (Askew and Shaw, 1986). Depending on the location of egg deposition and the feeding habits of the larvae, parasitoids are divided into

endoparasitoids, where eggs are laid inside the host and larvae feed on internal tissues, and ectoparasitoids, where eggs are deposited externally and the larvae feed on the host's body from the outside (Asgari and Rivers, 2011). Generally, ectoparasitoids tend to be idiobionts, while endoparasitoids are koinobionts (Jervis and Moe, 2012). Based on the number of mature eggs present at the time of emergence, parasitoids can also be classified into synovigenic species, where females emerge with only a few mature eggs, with more maturing over time as they acquire nutrients, and pro-ovigenic species, where all eggs are mature at the time of emergence (Jervis and Ellers, 2008). Additionally, depending on the number of parasitoids that successfully develop within a single host, they can be categorized as solitary parasitoids (one larva per host) or gregarious parasitoids (multiple larvae can develop within a single host) (Harvey et al., 2009). Various reproductive strategies are further differentiated, such as multiparasitism, where different species of parasitoids parasitize the same host; superparasitism, where a female lays eggs in a host already parasitized by another; and hyperparasitism, where a parasitoid lays its eggs in the larvae of another parasitoid species (van Alphen and Visser, 1990; Godfray and Hunter, 1992; Briggs and Collier, 2001).

Table 1-1. Classification of parasitoids with different life histories

Classification basis	Parasitoid type	Characteristics	Representative Species	References
Host physiology after parasitism	Idiobiont parasitoids	Host development stops after egg laying	*Leptopilina heterotoma*, *Leptopilina boulardi*, *Brachymeria podagrica*, *Dirhinus himalayanus*, *Dineulophus phthorimaeae*, *Dendrocerus carpenteri*	Otto and Mackauer, 1998; Savino et al., 2017; Brantley et al., 2021; Schuster and Sivakumar, 2024
	Koinobiont parasitoids	Host continues development after egg laying	*Cotesia vestalis*, *Aphidius ervi*, *Venturia canescens*, *Meteorus pulchricornis*, *Microplitis mediator*, *Pseudapanteles dignus*,	Xu et al., 2008; Harvey et al., 2017; McLean and Parker, 2020; Baghery and Michaud, 2024

			Lysiphlebus ambiguus	
Oviposition location	Endoparasitoids	Eggs laid inside the host	*Megalyridae*, *Braconidae*, *Cotesia vestalis*, *Diadromus collaris*, *Tetrastichus brontispae*, *Venturia canescens*, *Microplitis manilae*, *Meteorus pulchricornis*, *Microplitis mediator*, *Pseudapanteles dignus*	Harvey et al., 2017; Xing et al., 2023; Polaszek and Vilhemsen, 2023; Hu et al., 2024; Jiang et al., 2024; Baghery and Michaud, 2024
	Ectoparasitoids	Eggs laid outside the host	*Nasonia vitripennis*, *Theocolax elegans*, *Aroplectrus dimerus*, *Habrobracon hebetor*, *Bracon nigricans*, *Dineulophus phthorimaeae*, *Dendrocerus carpenteri*	Otto and Mackauer, 1998; Becchimanzi et al., 2020; Lepeco and Melo, 2022; Pers et al., 2023; Xiao et al., 2023; Polaszek and Vilhemsen, 2023; Yalemar et al., 2024; Baghery and Michaud, 2024
Egg maturity at emergence	Synovigenic parasitoids	Females emerge with immature or few mature eggs	*Trichogramma achaeae*, *Trichogramma brassicae*, *Anastatus fulloi*, *Anastatus japonicus*, *Mesocomys albitarsis*, *Mesocomys trabalae*,	Ueno and Ueno, 2007; Wang et al., 2014; Moiroux et al., 2018; Mu et al., 2023; Baghery et al., 2024;

			Aphidius ervi, *Neochrysocharis formosa*, *Itoplectis naranyae*, *Habrobracon hebetor*	Cabello et al., 2024;
	Pro-ovigenic parasitoids	Females emerge with all eggs fully mature	*Leptopilina boulardi*, *Leptopilina heterotoma*, *Asobara persimilis*, *Anagrus delicatus*, *Venturia canescens*, *Cotesia flavipes*, *Aphytis aonidiae*	Cronin and Strong, 1996; Rosenheim et al., 2000; Denis et al., 2012; Askari et al., 2020; Lemauf et al., 2021; Baghery et al., 2024;
Number of parasitoids emerging from a single host	Solitary parasitoids	Only one parasitoid emerges from a single host	*Brachymeria podagrica*, *Dirhinus himalayanus*, *Venturia canescens*, *Mesochorus gemellus*, *Encarsia formosa*, *Aphidius gifuensis*	Harvey et al., 2016; Liu et al., 2016; Zhang et al., 2018; Baghery and Michaud, 2024; Schuster and Sivakumar, 2024
	Gregarious parasitoids	Multiple parasitoids can emerge from a single host	*Pteromalus puparum*, *Aroplectrus dimerus*, *Habrobracon hebetor*, *Palmistichus elaeisis*, *Habrobracon gelechiae*, *Euplectrus separatae*	Nakamatsu and Tanaka, 2003; Daane et al., 2013; de S Pereira et al., 2017; Baghery and Michaud, 2024; Shi et al., 2022; Yalemar et al., 2024

3. Mating strategies of parasitoid wasps

For insects that reproduce through the combination of male and female gametes in the form of fertilized eggs, mating behavior plays a crucial role in the perpetuation

and evolution of populations, making it a key element of reproduction (Andersson and Simmons, 2006). In the case of sexually reproducing parasitoids, the mating behavior of females is directly linked to the sex ratio and quality of their offspring, ultimately determining the establishment and expansion of the population (Miyatake, 1997; Vahed, 1998; Hunter, 2001). Under natural selection, parasitoid wasps have developed a range of adaptive evolutionary strategies to cope with increasingly dynamic environments. To ensure the continued survival and success of their species, parasitoids have evolved diverse mating patterns that maximize the reproductive success of both parents and offspring (Shackelford and Goetz, 2006; van et al., 2024). The main adaptive mating strategies of parasitoids include multiple mating, inbreeding avoidance, and sexual selection.

3.1 The strategy and benefits of multiple mating in parasitoid wasps

Multiple mating is a common reproductive strategy observed in insects, involving two or more matings between a male and female pair or across different individuals (Arnqvist and Nilsson, 2000). The benefits of multiple mating in parasitoids occur on both individual and population levels. Numerous studies have demonstrated significant positive effects on parental traits such as oviposition period, longevity, fecundity, and offspring traits, including egg hatch rate, survival, and development time (Boulton and Shuker, 2015; Wang et al., 2021; Ramadan and Wang, 2021; Man et al., 2024). For males, multiple mating offers direct advantages, as it increases their reproductive fitness by producing a larger number of offspring. Most males can engage in multiple matings and the reproductive benefits they gain are influenced by the number of matings. Consequently, males have evolved various strategies to maximize their opportunities for multiple matings, which can be categorized into four main types (Vahed, 2015): 1) **Female guarding**: males occupy a territory where the species larvae are present and eliminate other males in the area. Once the virgin females emerge, the guarding male has the opportunity to mate with multiple females. For example, male *Asolcus basali* guard the host egg mass from which the females emerge and drive away other males to mate with the newly emerged females (Kuramitsu et al., 2019). 2) **Resource guarding**: males guard essential reproductive resources such as water, food, or oviposition sites. This attracts females to the site, where the male then mates with them. Male *Nasonia vitripennis* emerge before females and guard the hosts pupal case by biting holes through it. They establish territories around these openings to mate with emerging females (Leonard and Boake, 2006). 3) **Mating aggregations**: males gather in advantageous locations and compete for access to females. The strongest males display their dominance and mate with the attracted females. In *Hemipepsis ustulata*, males occupy specific landmark hills to exhibit their physiological dominance, thereby drawing more females for mating (Turchin, 1989). 4) **Mate searching**: this non-aggregative mating strategy involves males seeking out females in resource-rich environments where mating is likely to occur. In *Abispa ephippium* males search for females at water sources and mud patches, where females collect resources for nesting (Vahed, 2015).

Females also benefit from multiple mating, which can be classified into direct and indirect benefits, forming the basis for two hypothesis: the material benefits hypothesis and the genetic benefits hypothesis (Jeanne, 2001; Kenneth, 2002; Hosken and Stockley, 2003). 1) **Material benefits**: a, Nutritional benefits: males often provide nutritional resources during mating, which can enhance the females reproductive success. Quicke (1997) noted that sugar proteins or multinucleate cells from the male accessory glands might be transferred to the female and used for somatic maintenance or egg production. b, Compliance benefits: To avoid the cost of resisting mating or disturbance from other males, females may accept copulation if the cost of resistance exceeds that of compliance (Wang and Davis, 2006). 2) **Genetic benefits**: a, Selection of superior genes: Females can increase the genetic diversity of their offspring by selecting sperm from males with superior genetic traits. Multiple matings allow females to choose sperm from males that offer better genetic quality. In *Hemipepsis ustulata*, females tend to mate with dominant males that exhibit greater stamina and flight ability, resulting in higher-quality offspring (Thornhill and Alcock, 1983; Boulton et al., 2015). b, Sperm replenishment and replacement: Some females may not acquire enough sperm from a single mating to fertilize all of their eggs. Through multiple matings, females can replenish their sperm supply, increasing the number of fertilized eggs, or replace older, less viable sperm. This strategy leads to greater offspring production. Multiple matings by female *Encarsia sophia* significantly increase the oviposition period and parasitism rate on *Bemisia tabaci* (Man et al., 2024).

3.2 Strategies for inbreeding avoidance in parasitoid wasps

Inbreeding refers to the mating between closely related siblings, where the genotypes are identical or similar (Hedrick and Kalinowski, 2000; De et al., 2016). However, extensive research has demonstrated that inbreeding negatively impacts both parental and offspring fitness, leading to reduced reproductive success, a phenomenon known as inbreeding depression (Butcher et al., 2000; Vayssade et al., 2014). This effect is especially pronounced in parasitoids with single-locus complementary sex determination (sl-CSD), where heterozygous individuals develop as females, hemizygous individuals develop as males and homozygous individuals develop as diploid males that are either inviable or sterile (Ross et al., 1993; Heimpel and De Boer, 2008). When sibling mating occurs, there is a 50% chance of genetic incompatibility at the sl-CSD locus, leading to half of the offspring being sterile diploid males (Cowan and Stahlhut, 2004; Elias et al., 2009). Consequently, Hymenoptera species are more vulnerable to inbreeding depression than other diploid species (Zayed and Packer, 2005). To mitigate the detrimental effects of inbreeding depression and ensure population survival, species have evolved mechanisms to avoid inbreeding, a process known as inbreeding avoidance (Duthie and Reid, 2016; Wikberg et al., 2017). Parasitoids employ four main strategies to avoid inbreeding: 1) **Kin recognition**: The ability to distinguish between kin and non-kin is facilitated through specific cues, often olfactory or pheromone-based in insects. This distinction leads to differential behavioral responses towards kin, reducing the likelihood of

inbreeding (Gallot et al., 2020). Kin recognition is closely linked to mate choice and serves as a critical mechanism in inbreeding avoidance (Bollinger et al., 1991). Female *Venturia canescens* can differentiate between kin and non-kin males during courtship using olfactory cues (Metzger et al., 2010). 2) **Natal dispersal**: This strategy involves individuals leaving their birthplace shortly after emergence, before reaching sexual maturity, thus reducing the likelihood of encountering siblings for mating. Dispersal can be sex-biased, with either males or females predominantly dispersing (Huchard, 2017). In *Bracon hebetor*, inbreeding depression has been observed in laboratory mating trials, where inbred offspring result in sterile diploid males. However, in natural environments, sibling dispersal prior to mating reduces the chances of inbreeding (Ode et al., 1995). 3) **Reproductive suppression and delayed maturity**: Siblings from the same brood may reach sexual maturity at different times, preventing sibling mating. Early-maturing individuals will mate with non-related partners, while siblings are separated temporally or display differences in reproductive success when compared to unrelated individuals (Hoogland, 2013). In *Encarsia sophia*, a time gap of over 10 days exists between the hatching of male and female siblings, effectively reducing the likelihood of sibling encounters during mating (Man, 2020). 4) **Multiple mating**: in species with low dispersal rates, high levels of extra-pair mating occur. Females that mate with multiple males can acquire a variety of sperm and select the most compatible sperm for fertilization, thus reducing the negative effects of genetic incompatibility (Zeh and Zeh, 2010). In *Habrobracon hebetor*, multiple matings by females have been shown to reduce CSD load, thereby decreasing genetic incompatibility and improving offspring survival rates (Antolin et al., 2003).

3.3 Sexual selection strategies in mating of parasitoid wasps

Sexual selection refers to the process by which individuals of both sexes make choices to enhance mating success. This includes intrasexual competition, where individuals of the same sex compete for mating opportunities, as well as intersexual selection, in which individuals of the limited sex select partners from the opposite sex. Typically, this involves females assessing the quality of males and selecting the best potential mates (Boulton et al., 2015; O'Loughlin and Marcondes, 2024). For males, intrasexual competition for mating opportunities with females usually involves the following strategies: 1) males attempt to mate with as many females as possible to maximize their reproductive output; 2) males select high-quality females as mates to ensure better offspring; 3) males adjust the quality and quantity of sperm transferred based on the quality of the female and the intensity of sperm competition. When a male senses the presence of competitors, it may extend the duration of copulation to increase sperm transfer (Simmons, 2001; Wedell et al., 2002; Kelly, 2011; Bretman, 2011; Lane et al., 2015). For females, intersexual selection strategies when choosing high-quality males include: 1) accepting mating with the first encountered male without evaluating his quality; 2) accepting a previous male’s mating offer only if subsequent males are of lower quality; 3) only mating with males whose quality

exceeds an average threshold; 4) seeking out multiple males to ensure mating with the highest-quality male (Janetos, 1980; Wittenberger, 1983; Reall, 1990).

Additionally, sexual selection in parasitoids can be divided into pre-mating, intra-mating, and post-mating selection based on the timing and circumstances under which it occurs. 1) **Pre-mating** sexual selection primarily involves morphological traits such as body size, age and physiological characteristics related to mating experience. Larger males are more likely to succeed in mating competition and are preferred by females (Danielsson, 2001; Jimenez-Perez, 2004). Similarly, larger females, especially those with more substantial abdomens, tend to have an advantage in intrasexual competition for mates (Stuart-Smith et al., 2007; Busiere et al., 2008). Studies have shown that newly emerged, unmated individuals, particularly males with larger sperm packages and females with higher egg-laying capacity, are more likely to be selected as mates. For example, *Spalangia endius* females tend to prefer unmated males (Ivey et al., 2006; Xu and Wang, 2009; Lemaitre et al., 2009; King, 2010), while *Encarsia sophia* females prefer males with previous mating experience, possibly due to learned mating behaviors (Man et al., 2024). 2) **Intra-mating** sexual selection (sperm selection before fertilization) includes sperm competition and cryptic female choice. In environments with more females, males may increase their sperm investment by providing larger ejaculates to high-quality females, such as newly emerged, larger individuals, while conserving sperm for future mating opportunities. Females, in turn, engage in multiple matings to gain both material and genetic benefits, avoiding genetic incompatibility. This leads to sperm competition within the female's reproductive tract, where sperm from different males competes for fertilization (Wedell et al., 2002; Xu and Wang, 2014). Cryptic female choice allows females to control which male’s sperm is used after multiple matings (Dixson, 2002; Fedina, 2007). Research indicates that sperm competition follows one of three patterns: a) first-male sperm precedence, where sperm from the first mating male has the highest likelihood of fertilizing the eggs; b) last-male sperm precedence, where sperm from the last mating male is most likely to fertilize the eggs; c) no sperm precedence, where the order of mating does not influence fertilization success (Boomsma, 1996; Darwin, 2009). First-male sperm precedence has been observed in *Trichogramma euproctidis* (Damiens and Boivin, 2005; Martel et al., 2008b), while no sperm precedence has been found in *Habrobracon hebetor* (Ode et al., 1995), *Nasonia vitripennis* (Holmes, 1974), *Anisopteromalus calandrae* (Bressac et al., 2009), and *Diachasmimorpha longicaudata* (Martínez-Martínez et al., 1993). 3) **Post-mating** sexual selection occurs after fertilization and may involve strategies that prevent remating with other males, increasing reproductive success. For instance, in *Aphytis melinus*, males guard their mates to reduce the likelihood of further mating (Allen et al., 1994), while *Spalangia endius* males mark females with an anti-aphrodisiac substance post-mating to reduce their attractiveness to other males (King, 2010).

4. Oviposition strategies of parasitoid wasps

Oviposition is another crucial element of insect reproductive behavior. By employing various oviposition strategies, females optimize their reproductive success

(Godfray, 1993; Kafle et al., 2020). The parasitoid wasps primarily encompass host location and quality assessment, inter- and intraspecific competition, sex allocation strategies, and patch time allocation strategies.

4.1 Host location and quality assessment by parasitoid wasps

To maximize reproductive fitness, females must effectively locate suitable hosts and assess their quality. Various methods are employed by parasitoids to optimize their oviposition decisions and enhancing offspring survival. The search for hosts follows a sequence of stages, continuing until parasitoids are in close proximity to potential hosts (Vinson, 1976; Saunders et al., 2024). Different types of stimuli, such as chemical, visual and tactile cues are used to identify host locations (Jiang et al., 2024). The effectiveness of these cues depends on their reliability and detectability (Vet et al., 1991). Among them, chemical signals are a primary method for host location, as parasitoids can detect volatile organic compounds (VOCs) emitted by plants, particularly those released in response to herbivore damage. These volatiles guide parasitoids to host patches. For instance, cotton plants release specific volatiles when attacked by pests, which *Microplitis croceipes* detects and uses to locate suitable host larvae for oviposition (Morawo and Fadamiro, 2014a). Currently, researchers have focused their attention on another factor: the limitation of host quality on health status (Li et al., 2022; Chavarín-Gómez et al., 2023; Zhang et al., 2023; Aspin et al., 2024; Van Hee et al., 2024). "Host quality" refers to changes in the condition of the host that can affect the growth, development, and survival of parasitoids (Roberts et al., 2004; Betty, 2023). Studies have demonstrated that host quality is a critical factor in constraining parasitoid fitness. The growth and development rate of parasitoid larvae, as well as adult biomass, are significantly influenced by host size, age, and whether the host has already been parasitized (Holmes et al., 2023; Li et al., 2024).

4.1.1 Assessment of host size

The size of parasitoids is closely related to the size of their hosts, particularly in idiobiont parasitoids, as the host ceases to grow after parasitization, representing a fixed amount of resources (Xu et al., 2008; Sarikaya and Gülel, 2011). Therefore, larger hosts are generally more advantageous than smaller ones, as they provide more resources (Rivero and West, 2002). Females can gain adaptive benefits by laying unfertilized male eggs on larger hosts. In *Dinarmus basalis*, the size of males increases with host weight, reaching the maximum size for males. In most insect species, body size is often constrained by the number of offspring (Oksanen et al., 2003). Thus, larger males are able to produce more sperm, giving them a competitive edge in sexual selection. When females are not a limiting resource, larger males show at least a 37% advantage in offspring fitness over smaller males (Chevrier and Bressac, 2002). Additionally, body size has a more significant impact on the adaptive fitness of females than on males (Cloutier et al., 2000; Lacoume et al., 2006). Females tend to lay fertilized eggs on larger hosts to maximize their reproductive value (Yang et al., 2016).

4.1.2 Oviposition strategies based on host exploitation stage

When selecting a host, parasitoids consider not only the size of the host but also its age or developmental stage to determine whether to oviposit. Hosts at different developmental stages offer parasitoid larvae varying resources and environmental conditions, which in turn influence the growth and reproduction of the parasitoid. The latter typically uses host odor or other chemical signals to distinguish the developmental stage of the host, choosing hosts of different ages to maximize the survival and reproductive success of their offspring (Bell and Weaver, 2008). The age or developmental stage of the host significantly impacts the survival rate and development time of parasitoid larvae. For example, in *Meteorus pulchricornis*, older host larvae do not always provide better conditions, as larger hosts (such as L5 and L6) tend to have higher parasitoid larval mortality rates compared to smaller hosts (such as L2-L4). Additionally, the development time from egg to adult is longer in older hosts (L4-L6) than in younger ones (Harvey and Strand, 2002). Typically, younger hosts are preferred by parasitoids because their resources are more easily accessible and can be more effectively utilized by the developing parasitoid larvae (Ueno, 1997; Chen et al., 2024). Compared to older hosts, younger hosts present resources in forms that are more usable by parasitoid larvae. However, in some parasitoid species, such as *Nesolynx thymus*, *Aphidius ervi*, *Coccygomimus turionellae*, and *Trichopria* sp., a preference for older hosts has been observed. Although older hosts may provide fewer offspring, the resulting offspring tend to be larger, compensating for the reduced number. This strategy is a host utilization tactic commonly found in solitary parasitoids (Sandlan, 1982; Kumar et al., 1990; Sequeria and Mackauer, 1994; Aruna and Manjunath, 2009; Veena and Manjunath, 2013).

4.1.3 Distinguishing between healthy (unparasitized) and parasitized hosts

Parasitized hosts are generally regarded as low-quality resources, as they have already been exploited by other parasitoids and may no longer provide sufficient nutrients or space for the development of new parasitoid larvae. Consequently, many parasitoids exhibit host discrimination behavior when encountering parasitized hosts, opting to avoid them and instead seeking out unparasitized hosts for oviposition (van Alphen and Visser, 1990; Ruschioni et al., 2015; Hougardy et al., 2022). This behavior helps reduce intraspecific competition and enhances the survival rates of their offspring. Parasitoids employ various strategies to distinguish between parasitized and unparasitized hosts. One approach involves detecting external or internal chemical markers deposited by the ovipositing female on or inside the host. These chemical markers serve as cues that signal the parasitized status of the host (Nufio and Papaj, 2001; Stelinski et al., 2009). For example, *Leptopilina heterotoma* can detect the scent left by a competitor species, *Leptopilina clavipes*, and avoids hosts parasitized by *L. clavipes* (Janssen et al., 1995a; Tamò et al., 2006). Also, some parasitoids have limited ability to distinguish between parasitized and unparasitized hosts at close range. Instead, they rely on volatile organic compounds (VOCs) emitted by plants as long-range signals to identify whether a host has already been parasitized. *Microplitis croceipes* uses changes in plant VOC emissions caused by parasitism of the herbivore

Heliothis virescens to locate unparasitized hosts and avoid superparasitism (Kafle et al., 2020). Similarly, *Cotesia rubecula* distinguishes between unparasitized and parasitized hosts by detecting VOCs emitted from plants fed on by *Pieris rapae* larvae (Fatouros et al., 2005).

4.2 Competitive strategies in oviposition by parasitoid wasps

When parasitoids are unable to determine whether a host has already been parasitized or when non-parasitized hosts are difficult to locate within a short period, they may be forced to oviposit in hosts already parasitized by the same species (hyperparasitism) or by different parasitic species (multiparasitism) (Gandon et al., 2006; Ayala et al., 2022). Traditionally, superparasitism has been considered a result of erroneous oviposition by females, leading to wasted eggs and/or time resources (Gandon et al., 2006), thereby reducing health and increasing offspring mortality (Böckmann et al., 2012). However, recent studies have recognized that superparasitism may also function as an adaptive reproductive strategy when resources are scarce. In complex ecosystems, parasitoids adopt diverse oviposition strategies based on varying competitive pressures, such as engaging in aggressive behavior, opportunistic waiting, and reusing competitors' actions to respond to interspecific competition (Harvey et al., 2012; Mohamad et al., 2015; Pang et al., 2024). Different parasitoid species, when sharing resources, tend to adjust their strategies based on their competitive ability, host type and parasitic environment to enhance their survival and reproductive success. Under competition, *Dinarmus basalis* tends to engage in self-superparasitism to increase offspring numbers and gain an advantage in larval competition. It also exhibits aggressive behavior to prevent other species, such as *Eupelmus vuilleti*, from accessing the host (Mohamad et al., 2011). In contrast, *E. vuilleti* employs a "waiting strategy," waiting for its competitor to leave before reusing the host, attempting to remain undetected until gaining an opportunity for multiparasitism. This strategy proves advantageous in interspecific competition (Mohamad et al., 2012). These behaviors highlight the strategic differences parasitoids exhibit in response to various competitive scenarios.

Additionally, when hosts are scarce, especially when host resources are limited and the cost of continuing to search for non-parasitized hosts is high, parasitoids may choose to engage in superparasitism of already parasitized hosts to reduce search costs and sometimes provide adaptive benefits to their offspring (Roberts et al., 2004). In cases of host scarcity, *Venturia canescens* resorts to superparasitism, with a recorded success rate of 0.45 for the superparasitized offspring in winning the competition (Roberts and Schmidt, 2004). Superparasitism has been found to increase the body size of the offspring in *Aphidius ervi* and *Monoctonus paulensis*, offering adaptive benefits (Mackauer and Chau, 2001). Moreover, in both intraspecific and interspecific multiparasitism scenarii, parasitoids may adopt resource-sharing strategies, particularly when host resources are scarce or competition is intense. This can involve a variety of feeding behaviors or differential nutrient utilization to increase survival opportunities. For example, gregarious species *Glyptapanteles liparidis* and solitary *G. porthetriae* may both emerge from multiply parasitized *Lymantria dispar* larvae

(Marktl et al., 2002). When *Sesamia calamistis* larvae are rapidly subjected to multiparasitism, two gregarious endoparasitoid species, *Cotesia sesamiae* and *C. flavipes*, can emerge from the same host (Sallam et al., 2002). The larvae of the moth *Hadena bicruris* may simultaneously host the gregarious endoparasitoid *Microplitis tristis* and the ectoparasitoid *Bracon variator*. Due to the hemolymph-feeding behavior of *M. tristis*, sufficient resources remain within the host to allow a few *B. variator* parasitoids to continue developing (Elzinga et al., 2007).

4.3 Sex allocation strategies in oviposition by parasitoid wasps

Parasitoids exhibit a haplodiploid sex determination system, in which males are produced from unfertilized eggs, while females are produced from fertilized eggs. Consequently, females are able to precisely regulate the sex ratio of their offspring by controlling egg fertilization (West 2009; Wajnberg, 2012). Sex ratio adjustments are made by parasitoids based on the reproductive fitness they can achieve, with fitness being measured by the increase in the number of their second-generation offspring, which manifests as a ratio of males to females in the first generation (Hamilton, 1967). The well-known theory of local mate competition (LMC) has been proposed to explain sex ratio adjustments in parasitoids, where male siblings compete for mating resources. As competition intensifies, the mother invests more in producing daughters (Sean et al., 2002; West et al., 2003). This theory applies to the life history patterns of most parasitoid species, assuming that mated females establish themselves on host patches where their offspring will develop and mate before dispersing to find new patches. The LMC model predicts that female parasitoids should optimize the proportion of male offspring according to the number of females present in the host patch. Specifically, when “n” females settle on the same host patch, the optimal male ratio should be “(n-1)/2n” (Hamilton 1967; Gardner and Hardy, 2020; Abdi et al., 2020). This implies that females will increase the proportion of male offspring in patches with fewer competitors (i.e., where there are fewer females). During oviposition, females often lay male eggs first, followed by female eggs. This "male-first strategy" aligns with LMC model predictions and is considered an effective reproductive strategy, observed in species such as *Nasonia vitripennis*, *Melittobia australica*, and *Trichogramma chilonis* (Wajnberg 1993, 1994; Ivens et al., 2009; Tang et al., 2014). Additionally, some parasitoids adjust the sex ratio of their offspring based on host density. For instance, the male-to-female ratio in *Ooencyrtus kuvanae* decreases as host numbers increase, meaning that in patches with a higher density of hosts, the proportion of male offspring is lower (Hofstetter et al., 2024). Similarly, *Aphidius colemani* produces a higher proportion of female offspring as the density of its host, *Myzus persicae*, increases, enhancing parasitism efficiency (Khatri et al., 2021). Furthermore, recent studies have reported that a specialized type of parasitoid, heteronomous hyperparasitoids, also exhibit sex ratio adjustment capabilities. The sex ratio in *Encarsia sophia* offspring is adjusted according to the relative abundance of primary and secondary hosts. When secondary hosts are scarce (below 0.5) and host density increases, the sex ratio approaches 1:1 (Man et al., 2024). These strategies highlight the highly evolved reproductive adaptations of parasitoids in response to

varying ecological conditions and host availability, ensuring the maximization of offspring survival and reproductive success.

4.4 Patch time allocation strategies in oviposition by parasitoid wasps

A key decision that parasitoids must make during oviposition is how to optimize their time allocation within a host patch to maximize reproductive success. Since the time and resources available to parasitoids are typically limited, their patch time allocation strategy must strike an optimal balance between resource utilization and survival. The Marginal Value Theorem (MVT) can predict the balance between resource exploitation and survival for parasitoids, suggesting that foragers should leave a patch when the rate of resource acquisition falls below the environmental average, at which point they should search for a new patch (Charnov 1976; Haccou et al., 2003; Boivin et al., 2004; Wajnberg et al., 2012). This implies that parasitoids should depart when the likelihood of encountering hosts within a patch is lower than the average likelihood elsewhere. MVT also predicts that parasitoids should stay longer in high-quality patches containing more hosts available for attack and ensure that, before leaving, the marginal rate of resource use in different quality patches is equalized (Wajnberg, 2006). When provided with patches containing varying initial host numbers, the patch time allocation strategy of female *Trichogramma chilonis* appears to align with MVT predictions, as females tend to stay longer in high-quality patches to maintain a consistent offspring production rate (Wajnberg, 2006). Similarly, female *Anaphes victus* extend their residence time when both patch quality and travel time between patches increase (Boivin et al., 2004). Additionally, the presence of conspecific or interspecific competitors can modify a parasitoid's time allocation strategy, leading to different decisions in highly competitive environments. Foragers may choose to leave patches earlier when competitors are present to avoid resource depletion or direct competition (Wajnberg et al., 2004; Goubault et al., 2005). On the contrary, some parasitoids may stay longer when competitors are present, attempting to exploit resources once their rivals leave (Haccou et al., 2003; Hamelin et al., 2007). Female *Eupelmus vuilleti*, for instance, tend to wait in patch environments until *Dinarmus basalis* females depart before laying eggs (Mohamad et al., 2015).

5. Strategies of parasitoid wasps for suppressing host immunity

To successfully parasitize and complete their development within the host, parasitoids must first overcome the host’s immune defenses. Throughout their long-term coevolution with hosts, parasitoids have evolved a series of strategies to regulate or adapt to host immune responses. These strategies can be classified into active suppression and passive evasion (Brantley et al., 2024; Cinege et al., 2024; Zhou et al., 2024). Active suppression refers to the inhibition of the host immune system by parasitoid factors, which prevent the host's immune functions from operating normally. Passive evasion, on the other hand, involves avoiding attacks from the host's immune system, such as by masking their eggs or embryos with surface components to evade

the host's "non-self" recognition or by laying eggs in locations that the host immune system cannot reach (e.g., ganglia or midgut), or during periods when the host's immune capacity is weaker (e.g., during the egg stage) (Beckage and Gelman, 2004; Kraaijeveld and Godfray, 2009). In overcoming host immunity, parasitoids primarily rely on parasitic factors that they introduce into the host during oviposition or larval development. These factors manipulate critical physiological processes within the host, ensuring successful parasitization and the normal development of their offspring (Pennacchio and Strand, 2006; Asgari and Rivers, 2011; Moreau and Asgari, 2015). These parasitic factors include polydnaviruses (PDVs) (Gao et al., 2022), venom (Ye et al., 2024), virus-like particles (VLPs) (Cerqueira et al., 2022), ovarian proteins (Salvia et al., 2023) and teratocytes, which are released by embryos or larvae (Pinto et al., 2022). These factors play crucial roles in subduing host immunity and ensuring the successful development of the parasitoid larvae (Richards and Edwards, 2002; Richards, 2012) (Table 1-2).

5.1 Strategies of parasitoid wasps for evading host immunity

In their strategy to evade host immunity, parasitoids deposit their eggs into specific host tissues that are inaccessible to the host's immune system, thus preventing the eggs from being recognized by the host (Eslin et al., 1996; Schmidt et al., 2005). Alternatively, molecular mimicry is employed (Rotheram, 1973; Feddersen et al., 1986; Suzuki and Tanaka, 2006), allowing the parasitoid eggs to avoid detection by the host. For example, *Aulosaphes constractus* deposits its sticky eggs into host tissues such as the final segment of muscles, Malpighian tubules, digestive system, fat body, neural chain, or subdermal tissue areas where host hemocytes or effector molecules cannot reach, thus avoiding host immune recognition (Eslin et al., 1996; Prevost et al., 2005). The localized immune evasion strategies of parasitoids help protect their offspring without triggering the host's immune response. For instance, when *Macrocentrus cingulum* parasitizes *Ostrinia furnacalis*, the host's hemocyte count, morphology, and behavior remain unchanged, although its encapsulation ability against microbeads increases (Hu et al., 2003). This is likely related to protective factors on the egg surface (Hu et al., 2014). The egg surface of *Cotesia rubecul***a** adheres to a calyx fluid protein that passively shields it from encapsulation by *Pieris rapae* hemocytes before PDV gene expression begins (Asgari et al., 2011). Similarly, *Campoletis chlorideae* eggs passively evade encapsulation by *Helicoverpa armigera* larvae through surface components (Han et al., 2013). Recently, it was observed that *Pteromalus puparum* larval saliva increases hemocyte death rates, thereby suppressing melanization in host hemolymph (Shi et al., 2022).

5.2 Strategies of parasitoid wasps for actively suppressing host immunity

5.2.1 Cellular immunity

Through long-term coevolution, parasitoids have developed a set of counter-defense strategies to overcome the host's immune system. Insects primarily employ plasmatocytes, granulocytes, and oenocytoids to engage in phagocytosis, nodulation,

and encapsulation of foreign entities (Hillyer et al., 2002; Wang et al., 2023). Larger foreign bodies, such as parasitoid eggs, are mainly killed through encapsulation by suffocation. Parasitoids use parasitic factors to alter the number and ratio of host hemocytes, inducing cell lysis and apoptosis while inhibiting hemocyte spreading and adhesion capabilities, ultimately weakening the host’s encapsulation response and evading attack (Lanzrein et al., 1998). For example, the P4 protein in the venom of *Leptopilina boulardi* can alter the quantity and characteristics of *Drosophila* hemocytes and regulate the expression of a gene similar to *collier* (Crozatier et al., 2004; Labrosse et al., 2005). Schlenke et al. (2007) found that after parasitization, the expression levels of two genes controlling hemocyte proliferation in *Drosophila melanogaster* were increased. Additionally, calreticulin in the venom of *Cotesia rubecula* was found to inhibit the spreading of host hemocytes (Zhang et al., 2006).

5.2.2 Humoral immunity

In addition to cellular immunity, humoral immunity plays a crucial role in protecting organisms from foreign invaders. Three common immune factors include antimicrobial peptides (AMPs), phenoloxidase (PO), and lysozyme (Fang et al., 2016; Zhou et al., 2023; Vesala et al., 2024). When hosts are parasitized, antimicrobial peptides and lysozyme are rapidly produced within the host. For example, when *Eretmocerus mundus* larvae penetrate *Bemisia tabaci*, the host's Knottin gene transcription is significantly upregulated to combat the parasitism (Mahadav et al., 2008). Microarray analysis reveals that genes involved in the Toll and JAK/STAT pathways, such as *dome*, *hop*, *nec*, and *TI*, exhibit differential expression following parasitism in *Drosophila*. The Toll and JAK/STAT pathways are common immune signaling pathways in insects, involving a series of interconnected signaling molecules. In addition, the Imd and JNK pathways also play a role in insect immunity. These pathways are capable of inducing the production of antimicrobial peptides and defensins, but activation requires specific recognition of pathogen-associated molecular patterns (Yu et al., 2022; Aalto et al., 2023).

The inhibition of host humoral immunity by parasitoids primarily manifests through the regulation of hemolymph melanization in the host (Liu et al., 2018; Yang et al., 2020; Wang et al., 2021). The melanization reaction is a complex cascade involving many serine proteases. Phenoloxidase, the end product of this cascade, oxidizes substances such as tyrosine, dopa, and dopamine into melanin, which encapsulates and kills the parasitoid eggs (Asgari et al., 2003; Wu et al., 2020). Parasitoids can modulate the transcriptional levels of genes related to the melanization response. After parasitism by *D. melanogaster* and *Archips oporanus*, the transcriptional levels of phenoloxidase genes in the hosts were differentially expressed (Doucet et al., 2010; Yang et al., 2020). Transcriptomic sequencing of *Bemisia tabaci* parasitized by *Eretmocerus mundus* revealed that the transcription of serine protease inhibitor (Serpin) genes in the host was suppressed, reducing the occurrence of melanization (Mahadav et al., 2008). Additionally, Mahadav and Oliver (2003) observed that parasitism of whiteflies and aphids by parasitoids led to the proliferation of symbiotic bacteria within the hosts, which correspondingly reduced the emergence rates of the

parasitoids. The symbiotic bacteria enhanced the host's resistance to parasitism (Oliver et al., 2003; Mahadav et al., 2008).

Table 1-2. Defensive strategies of parasitoid wasps against host immunity

Immune defense strategy	Parasitic factors	Representative species	References
Active defense	Venom	*Nasonia vitripennis*, *Habrobracon hebetor*, *Leptopilina boulardi*, *Leptopilina heterotoma*, *Asobara japonica*, *Trichopria drosophilae*, *Pachycrepoideus vindemmiae*, *Cotesia chilonis*, *Theocolax elegans*, *Pteromalus puparum*	de Graaf et al., 2010; Yan et al., 2017; Ye et al., 2022; Xiao et al., 2023; Kryukova et al., 2024; Yang et al., 2024
	Polydnaviruses (PDVs)	*Cotesia bracovirus*, *Microplitis manilae*, *Chelonus formosanus*, *Snellenius manilae*, *Venturia canescens*, *Glyptapanteles indiensis*, *Chelonus inanitus*	Serbielle et al., 2012; Leobold et al., 2018; Tang et al., 2021; Yuan et al., 2022; Gulinuer et al., 2023; Wang et al., 2023
	Teratocytes	*Cotesia flavipes*, *Cotesia vestalis*, *Aphidius ervi*, *Cotesia plutellae*, *Toxoneuron nigriceps*, *Encarsia pergandiella*, *Microplitis croceipes*	Consoli et al., 2007; Strand, 2014; Mancini et al., 2016; Salvia et al., 2019; Pinto et al., 2023; Wu et al., 2023
	Virus-like particles (VLPs)	*Leptopilina heterotoma*, *Leptopilina boulardi*, *Venturia canescens*, *Microctonus aethiopoides*, *Opius concolor*, *Leptopilina victoriae*	Jacas et al., 1997; Morales et al., 2005; Barratt et al., 2006; Reineke et al., 2006; Gueguen et al., 2011
Passive defense	Ovarian proteins	*Toxoneuron nigriceps*, *Cotesia*	Tanaka et al., 2002; Dorémus et

		chilonis, *Hyposoter didymator*, *Cotesia kariyai*, *Macrocentrus cingulum*	al., 2013; Yin et al., 2018; Teng et al., 2019; Salvia et al., 2022
	Larval embryonic secretions	*Macrocentrus cungulum*, *Pimpla turionellae*, *Copidosoma floridanum*	Uka et al., 2006; Hu et al., 2014; Kaya et al., 2021

6. Nutritional utilization strategies in parasitoid wasps reproduction

The nutritional utilization strategies of parasitoids are designed to maximize reproductive success (fitness) by effectively acquiring and distributing limited nutritional resources. Under constraints of limited energy reserves and external environmental factors, parasitoids adjust resource allocation strategies to optimize their fitness. Parasitoids balance trade-offs between immediate reproductive gains and long-term survival benefits when foraging, choosing resources that favor either current reproduction or an extended lifespan, such as breeding sites, mates, or food sources (Wolf et al., 2007; Damien et al., 2019). They utilize various nutritional sources, including nectar, extrafloral nectaries, honeydew, and nutrients from the host, such as hemolymph and tissue, to support adult survival and reproduction. These diverse resource sources are crucial, especially when food is scarce, helping parasitoids maintain fertility and vitality (Hu et al., 2024). These nutritional sources can be categorized into host-derived and non-host-derived nutrients (Table 1-3).

6.1 Host nutritional utilization strategies

The nutrition derived from hosts by parasitoids occurs through two main pathways: parasitism during the larval stage and feeding during the adult stage. Depending on whether feeding and parasitism occur on the same host, parasitoid feeding behavior can be classified as either simultaneous or non-simultaneous. Additionally, based on whether feeding leads to host death, parasitoid feeding can be categorized as lethal or non-lethal (Jervis and Kidd, 1986). Lethal feeding refers to the behavior where the host dies directly after being fed on by the parasitoid, whereas non-lethal feeding allows the host to survive after being fed on. Non-lethal feeding can be either simultaneous or non-simultaneous (Zhang et al., 2022). Parasitoid larvae adopt different feeding strategies within the host, which can be divided into tissue feeding and hemolymph feeding. Tissue feeders consume most of the host's tissues, whereas hemolymph feeders take in only the host's hemolymph and fat bodies. This allows hemolymph feeders to more flexibly exploit host resources and minimize conflicts with predators (Foti et al., 2017; Harvey and Gols, 2018). Most studies suggest that hemolymph feeders have an advantage over tissue feeders. Hemolymph is rich in nitrogen compounds, carbohydrates, and lipids, which serve as important nutrients for

parasitoids, and it allows females to better cope with host quality variations, with less impact from changes in host quality (Gauld and Bolton, 1988; Quicke, 2015). For example, hemolymph-feeding species of the genera *Cotesia* and *Microplitis* can exploit a wide range of host stages and use the host as a bodyguard against predators until the host's death (Harvey and Malcicka, 2016). Additionally, some parasitoids engage in dual feeding behavior, consuming hemolymph inside the host and tissue outside the host. This strategy enhances their ability to utilize nutritional resources under varying environmental conditions (Harvey, 2005).The aphid wasp *Aphelinus asychis* feeds not only on the host's hemolymph but also on its gut contents, allowing it to adapt to complex and changing host environments (Cate et al., 1974).

6.2 Non-host nutritional utilization strategies

Parasitoids also supplement their carbohydrate and energy reserves by consuming non-host food sources such as honeydew, nectar, extrafloral nectar, and plant exudates. These food sources provide essential sugars and amino acids, extending adult longevity and enhancing oocyte maturation and reproductive success (Wäckers and van Rijn, 2005; Heimpel and Jervis, 2005; Straser et al., 2023). Honeydew, which is excreted by hemipteran insects (e.g., aphids, whiteflies, planthoppers, and scale insects) after feeding on plant sap, primarily consists of disaccharides such as trehalose and trisaccharides like melezitose (Heimpel et al., 2004). Compared to honeydew, nectar and extrafloral nectar are more easily observed and utilized, providing parasitoids with greater access to these resources in the field (Gilbert & Jervis, 1998; Straser et al., 2024; Gurr et al., 2024). The use of floral nectar by parasitoids largely depends on flower structure and nectar concentration, making it an important source of carbohydrates and sugars (Wäckers, 2004). However, due to competition from other species, the proportion of nectar that parasitoids can access is relatively low (Pritsh, 1993). In some cases, parasitoids also obtain energy from plant surface exudates or fruit juices, which are rich in carbohydrates. *Phanerotoma franklini* has been observed feeding on exudates from cranberry leaves (Sisterson and Averill, 2002), and *Asobara* sp. have been reported feeding on fermented fruit juices (Eijs et al., 2010). By supplementing their diets with a variety of nutritional sources, parasitoids demonstrate longer oviposition periods and increased offspring production (Ellers et al., 2011; Tena et al., 2018).

6.3 Adaptive regulation of nutrient acquisition and allocation

Parasitoids exhibit a high degree of adaptability in nutrient acquisition and allocation. The nutrients they obtain can be used for oviposition (Heimpel et al., 2005), maintaining survival (Chan and Godfray, 1993), or simultaneously supporting both egg production and survival (Williams and Roane, 2007). When parasitoids fall below a certain energy threshold or critical oviposition level, they tend to feed on the host without laying eggs inside it. In such cases, the parasitoids likely allocate their internal resources according to the mentioned principles (Chan and Godfray, 1993). From a population dynamics perspective, the stability of the host-parasitoid system may depend on whether parasitoids have a metabolic need for nutrients derived from feeding on the host (Chen et al., 2016). Furthermore, when parasitoids are able to

utilize nutrients obtained from host feeding for both survival and reproduction, non-host food resources may similarly affect the host-parasitoid system (Tena et al., 2016). parasitoids are capable of adjusting nutrient allocation between host and non-host resources under varying environmental conditions to optimize reproductive success and survival. For instance, when host resources are scarce, parasitoids adjust their nutrient allocation strategies to balance survival and reproduction (Jervis et al., 2008; Ellers et al., 2011). In conditions of host scarcity, some parasitoids can reabsorb their eggs or reallocate oocytes to obtain the necessary nutrients for sustaining survival and adaptability, thereby optimizing their reproductive success (Hougardy et al., 2005; Carneiro et al., 2009). *Pimpla turionellae* exhibits a unique capacity for resource utilization under host deprivation by maintaining continuous oviposition through muscle reabsorption until egg reabsorption begins (Sandlan, 1979; Jervis et al., 2005). This adaptive regulatory mechanism allows parasitoids to effectively utilize available resources in dynamic environments.

Table 1-3. Nutritional sources of parasitoid wasps and their effects on reproduction

Nutrition type	Source	Main substances provided	Representative species	Impact on fitness	References
Host	Tissues	Carbohydrates, amino acids, lipids	*Campoletis sonorensis*, *Dolichogenidea sicaria*, *Toxoneuron nigriceps*	Supports larval development, usually results in larger body size	Gauld and Bolton, 1988; Falabella et al., 2003; Pennacchio et al., 2014; Quicke, 2015
	Hemolymph	Carbohydrates, amino acids, lipids, nitrogen compounds	*Microplitis croceipes*, *Cotesia vestalis*, *Cotesia kariyai*, *Microplitis demolitor*	Typically utilized by gregarious parasitoids, unaffected by host age	Nakamatsu and Tanaka, 2002; Pruijssers et al., 2009; Harvey and Malcicka, 2016
Non-host	Honeydew	Carbohydrates, sugars	*Aphidius gifuensis*, *Diadegma insulare*, *Trichogramma dendrolimi*, *Encarsia formosa*	Increases adult longevity and enhances female fecundity	Siekmann et al., 2001; Lee et al., 2004; Tena et al., 2015; Benelli et al., 2017; He et al.,

					2018; Ayelo et al., 2022; Xu et al., 2024
	Nectar	Carbohydrates, sugars	*Cotesia glomerata*, *Heterospilus prosopidis*, *Pimpla turionellae*, *Hadronotus pennsylvanicus*	Increases adult longevity and enhances female fecundity	Siekmann et al., 2001; Wäckers, 2004; Tena et al., 2015; Benelli et al., 2017; Jones et al., 2017
	Pollen/Plant Juice	Carbohydrates, sugars	*Spalangia cameroni*, *Lysibia nana*, *Gelis agilis*, *Phanerotoma franklini*	Increases adult longevity and enhances female fecundity	Sisterton and Averill, 2002; Bernstein and Jervis, 2006; Harvey et al., 2012; Taylor et al., 2022

7 Conclusion

In behavioral ecology, strategys refers to a set of decision-making rules that have evolved and are genetically based. For parasitoid wasps, reproductive behavioral strategies are one of the key features in their life history evolution, reflecting their adaptive responses to hosts and habitat environments. The core of these reproductive strategies lies in how parasitoids effectively utilize their resources to maximize reproductive output. During reproduction, parasitoids face two main pressures: physiological pressures, including their egg load, vitality, mobility, and accumulated experience; and environmental pressures, such as the presence of competitors, host distribution and density, and host patch utilization (Hubbard et al., 1987; van Alphen and Visser, 1990; Krüger et al., 2024). According to the principle of reproductive fitness, organisms must balance the allocation of energy between reproduction and survival (Shuker and West, 2004). Excessive investment in current reproduction may reduce future reproductive opportunities. Thus, under the long-term pressures of natural selection, organisms develop optimal energy allocation strategies to maximize reproductive fitness. Parasitoids ultimate reproductive fitness is primarily determined by the allocation of sperm/eggs, host utilization, and the development of their offspring (Charnov et al., 1981; Waage and Lane, 1983; Charnov and Skinner, 1984).

By summarizing the diverse reproductive strategies of parasitoids in mating, oviposition, host immune defense and feeding, it has been revealed how parasitoids maximize their reproductive success in dynamic environments. Throughout their evolutionary history, parasitoids have developed flexible reproductive behaviors in response to changes in external environmental factors and internal physiological states. Parasitoids with varied ecological traits exhibit a range of reproductive strategies. For example, in gregarious parasitoids, females often engage in multiple mating to avoid competition for mates, while solitary parasitoids typically mate only once (Chevrier and Bressac, 2002). Solitary parasitoids generally produce male offspring in smaller hosts and female offspring in larger hosts (Donaldson and Walter, 1984; Mendel, 1986; King, 1989), whereas this strategy is not applicable to gregarious parasitoids. In gregarious species, reproductive success is not only influenced by host size but also by the number of offspring developing within the host (Luck et al., 1982; Hardy et al., 1992; Gu et al., 2003). Idiobiont parasitoids often demonstrate a preference for laying eggs in larger hosts (Takagi, 1985; Hardy et al., 1992; Zaviezo and Mills, 2000; Bezemer and Mills, 2003), whereas koinobiont parasitoids do not adjust their oviposition strategy based on host size, as the size of the host at the time of parasitism does not accurately represent the final nutritional resources available to the offspring (Werren, 1984; Waage, 1986; Sequeira and Mackauer, 1992; Godfray, 1994; Bukovinszky et al., 2009). In idiobiont parasitoids, host size also influences sex ratio adjustment strategies, but this is less common in koinobionts. Since koinobiont hosts continue to grow after parasitism, parasitoids find it difficult to predict the final size of the host, and thus do not typically adjust the sex ratio of their offspring based on host size (King and King, 1994; King and Lee, 1994). In pro-ovigenic parasitoids, nutrient intake is primarily directed towards maintaining physiological functions, survival, and body development, resulting in a strong body structure and enhanced dispersal capabilities, which give them a clear advantage in host-searching abilities. In contrast, synovigenic parasitoids allocate a significant portion of the nutrients obtained to egg development and maturation, and their reproductive capacity cannot be enhanced after the initial stages of adulthood. Some highly synovigenic parasitoids exhibit aptery, a trait that reduces the likelihood of egg limitation, particularly in the later stages of life, where this advantage becomes more pronounced (Jervis et al., 2008).

Current research on the reproductive fitness of parasitoid wasps has provided a solid foundation, yet several issues remain that require further investigation. Firstly, most existing studies focus on individual behaviors of parasitoids, often overlooking the interactions between behaviors in complex natural environments. In reality, different behavioral strategies frequently influence each other, making it essential to consider multiple strategies simultaneously for adapting to dynamic environments. Besides the biological factors discussed in this paper, abiotic factors such as temperature, humidity, and light intensity also affect parasitoid reproductive capacity to varying degrees (Jervis et al., 2008; Nufio and Papaj, 2001). Future research needs to increase the ecological complexity of experiments to more comprehensively understand the various constraints influencing the evolution of parasitoid reproductive strategies in

natural conditions and their relative importance (Harvey, 2005). Secondly, there is a lack of comprehensive metrics to measure parasitoid reproductive fitness, as fitness is influenced by several factors such as parental lifespan, oviposition capacity, and offspring survival rate, which may have positive or negative interactions. Additionally, while parasitoids are small in size and the application of new-generation technologies in parasitoid research has been relatively slow, advancements in technology hold promise for revealing the genetic and adaptive mechanisms behind their reproductive strategies and behaviors through multi-omics approaches. Future studies should further explore the adaptive mechanisms of parasitoids in diverse ecosystems and how they achieve optimal reproductive strategies under varying environmental conditions. In particular, research on parasitoid behavioral adaptations and physiological adjustments in response to climate change will help to uncover the effects of climate change on parasitoid population dynamics and biological control potential. Moreover, gaining a deeper understanding of the role parasitoids play in controlling pest populations and maintaining ecological balance will provide important theoretical and practical support for developing more sustainable pest management strategies. By integrating research approaches from behavioral ecology, evolutionary biology, and molecular biology, the adaptive mechanisms of parasitoids can be further elucidated, paving the way for more effective and sustainable biological control techniques. Strengthening the understanding of parasitoid interactions with their hosts and environments will enhance the effectiveness of parasitoids in natural pest control applications.

Chapter 2

Problematic, research aim, thesis outline, and experimental design

"*In the world of insects, nothing is as it seems*."

Eraldo Banovac

1. Problematic

Bemisia tabaci is the only insect to date that has been labeled a "super pest," and biological control has proven to be an effective method against it. Among the dominant parasitoids used in biological control are species of *Encarsia* wasps, such as *Encarsia sophia*. These wasps are hyperparasitoids with a unique reproductive mode in which males and females develop differently. Females develop inside whitefly nymphs, referred to as the primary hosts, while males develop on wasp larvae that are already present within the whitefly nymphs, known as the secondary hosts. Due to this unique reproductive mode, in a new habitat where secondary hosts may be scarce or absent, the population of these wasps may become highly female-biased, potentially preventing them from producing offspring altogether. Given this risk to population establishment, the questions arise: How do heteronomous hyperparasitoids succeed in producing males and establishing populations under such conditions, and what advantages allow them to persist without being eliminated?

2. Research aims

This project aims to explore the host adaptability mechanisms of the heteronomous hyperparasitoid wasp, a unique type of parasitoid, using methods from insect behavior, insect physiology, molecular biology, transcriptomics, and genomics. The thesis is divided into several objectives:

- Studying the sex allocation mechanisms of *Encarsia sophia* in different host environments
- Sequencing and assembly of the chromosome-level genome of *Encarsia sophia*
- Investigating the decision-making mechanism of heteronomous oviposition by *Encarsia sophia* in distinguishing between primary and secondary hosts

3. Thesis outline

The following experimental chapters are designed based on the insights provided in **Chapter 1,** aiming to fill the research gap in understanding the ability of heteronomous parasitoid wasps to adapt to complex host environments.

Chapter 3 examines the sex allocation mechanisms of heteronomous hyperparasitoids in different host environments, with the goal of understanding the species' population adaptability.

Chapter 4 provides the genome of the first heteronomous hyperparasitoid species globally and explores the divergence time and gene family evolution compared to primary parasitoid species at the genomic level.

Chapter 5 investigates the heteronomous oviposition mechanism of heteronomous hyperparasitoids, aiming to clarify the intra- and interspecific competition abilities of this species.

Finally, **Chapter 6** offers a general discussion and summary of all notable findings, providing suggestions, opinions, and perspectives to contribute to the future large-scale production and field application of heteronomous hyperparasitoids.

4. Experimental design

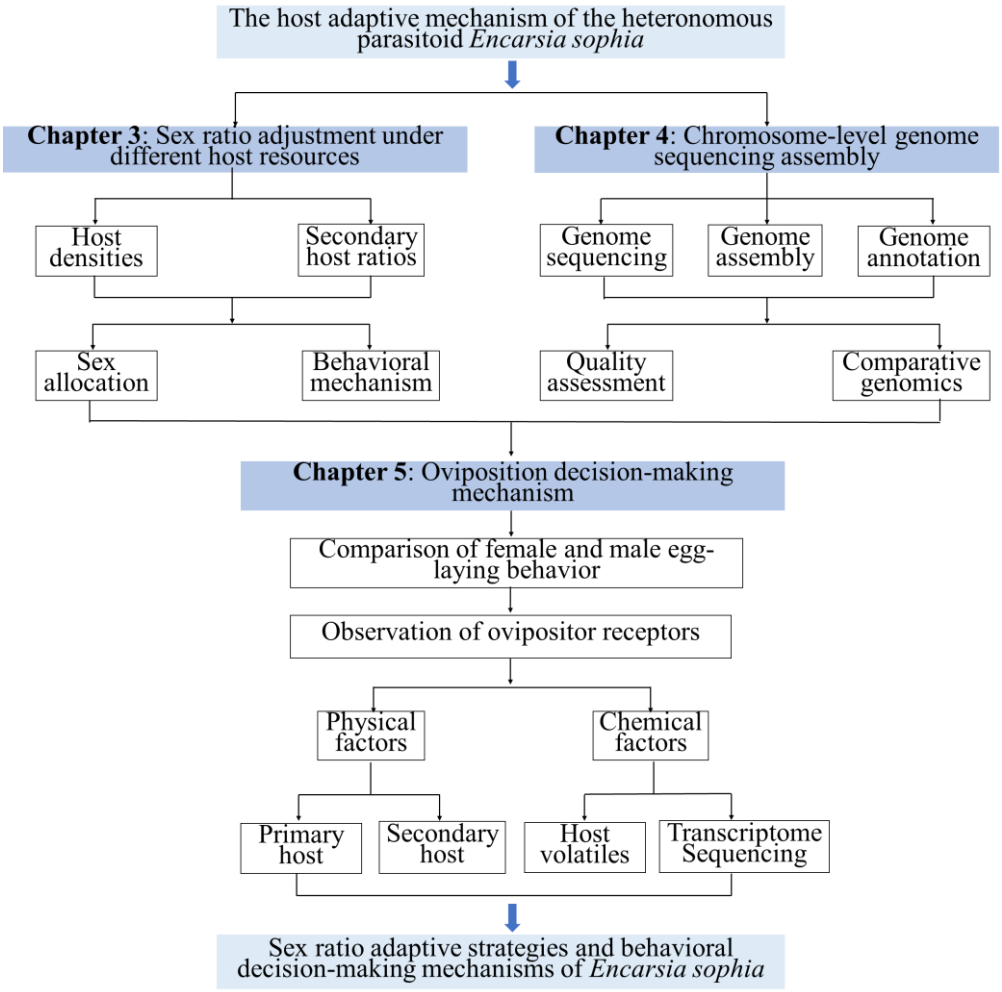


Figure 2-1. Technical route of the project "The host adaptive mechanism of the heteronomous parasitoid *Encarsia sophia*".

Chapter 3

Can heteronomous hyperparasitoids recognize host abundance and adjust offspring ratio?

"*The insect world is nature's most efficient and fascinating machine*."

E.O. Wilson

Adapted from:

Man, X., Sun, L., Francis, F., Yang, N., Liu, W. Can heteronomous hyperparasitoids recognize host abundance and adjust offspring ratio? *Entomologia Generalis*, 2024. 44(4), 1017-1025 https://doi.org/10.1127/entomologia/2024/2508

Abstract

Sex ratio is crucial in the reproductive dynamics of bisexual insects. In the Aphelinidae family, heteronomous hyperparasitoids like *Encarsia sophia* show distinct behaviors where females (from fertilized eggs) target primary hosts, and males (from unfertilized eggs) parasitize secondary hosts. This sex determination pattern means that host resource abundance significantly impacts sex ratio, affecting population dynamics. However, the sex distribution of these parasitoids remains a topic of debate. This study examined *E. sophia* targeting *Bemisia tabaci*, adjusting host densities (30, 50, 70 hosts/9.6 cm^2) and secondary host ratios (0.2, 0.5, 0.8). Females were observed for recognition of varying host conditions and adjustments in offspring sex ratio and behavior. When ratio of secondary hosts surpassed that of primary hosts or in cases of low host density (host limitation), *E. sophia*'s offspring sex ratio adapted according to the relative abundance of primary and secondary hosts. Conversely, with low secondary host ratios (<0.5) and higher host density, the sex ratio approached 1:1. Observations showed females quickly perceived host density, increasing oviposition on secondary hosts with higher density, resulting in more males, and increasing feeding on primary hosts, reducing female offspring. Importantly, by examining oviposition and feeding under different host resource conditions, we identified the optimal rearing strategy: a secondary host ratio of 0.2 and a host density of 30 hosts/9.6 cm^2. This study not only introduces the sex ratio theory for heteronomous hyperparasitoids but also provides a framework for more accurately assessing their environmental adaptability and for large-scale production.

***Keywords*:** smart livestock farming, animal welfare, thermal comfort, group measurement, behavioural index

1 Introduction

Heteronomous hyperparasitoids in the Aphelinidae family are a special type of parasitoid wasps, primarily including genera such as *Coccophagus*, *Coccobius*, *Coccophagoides*, and *Encarsia* (Hunter and Woolley, 2001). They play a significant role in the biological control of many major pests(Tize et al., 2023; Zhang et al., 2023; Shahbazvar et al., 2022). Similar to other haplodiploid insects, autoparasitic wasps evolve from unfertilized haploid eggs into males and fertilized diploid eggs into females. The distinction lies in the fact that female wasps originate from fertilized eggs, developing as primary parasitoids of Hemiptera nymphs, while male wasps stem from unfertilized eggs, evolving into secondary parasitoids (hyperparasitoids) on wasp larvae or pupae within Hemiptera nymphs (Walter, 1983; Godfray and Hunter, 1992; Hu et al., 2010). Heteronomous hyperparasitoids have the potential to induce mortality in conspecific or heterospecific primary parasitoid larvae, thus giving rise to lethal interference competition. This phenomenon has spurred controversy in biological control and attracted significant scientific attention(Xu et al., 2018; Kidane et al.,2020; Zhao et al., 2022). Nevertheless, based on the reproductive characteristics of heteronomous hyperparasitoids, lethal interference competition primarily manifests during the reproduction process of male offspring. Consequently, the sex ratio distribution of heteronomous hyperparasitoids emerges as a pivotal factor influencing their biological control effectiveness.

For heteronomous hyperparasitoids, they reproduce male offspring using larvae of conspecific or heterospecific primary parasitoids. The abundance of host resources and the diversity of related parasitoids directly influence the regulation of offspring sex ratios (Colgan and Taylor 1981; Godfray and Waage 1990; Bon et al., 2022). Therefore, does the sex ratio of heteronomous hyperparasitoid offspring depend solely on the type of host? Fisher (1930) introduced the foundational theory of sex ratio regulation in species. He posited that, in a large population with random mating between sexes, parents should invest equivalent resources in both male and female offspring, resulting in an offspring sex ratio of 1:1. However, the applicability of this theory to heteronomous hyperparasitoids and the mechanisms governing sex ratio adjustment remain contentious (Fisher, 1930; Harvey et al., 2013). Godfray extended Fisher's sex ratio regulation theory to heteronomous hyperparasitoids, proposing a sex ratio regulation mechanism under conditions of host and egg limitation. In cases of abundant host resources (egg limitation), Godfray argued that the offspring sex ratio of heteronomous hyperparasitoids is 1:1. Conversely, in situations with a lack of host resources (host limitation), the offspring sex ratio is determined by the relative abundance of primary and secondary hosts (Godfray and Waage, 1990; Godfray and Hunter, 1992, 1994). Diverging from Godfray's perspective, Walter and Donaldson disputed the applicability of Fisher's theory to sex adjustment in heteronomous hyperparasitoids. They contended that the sex ratio of heteronomous hyperparasitoid offspring is not 1:1 when host resources are sufficient. According to their viewpoint, female wasps adopt a fixed reproductive strategy, producing offspring of the corresponding sex based on the type of host, irrespective of host resource abundance.

Then, the offspring ratio of heteronomous hyperparasitoids, whether in resource-rich or resource-limited conditions, is linked to individual reproductive behavior and the relative abundance of the two hosts (Walter and Donaldson, 1994). The central focus of the debate between Godfray and Walter regarding the theory of sex ratio regulation revolves around whether, as the host abundance increases, the offspring sex ratio of heteronomous hyperparasitoids is dictated by the ratio of primary hosts to secondary hosts or tends towards 1:1.

In the mass rearing of Hymenoptera parasitoids, the overproduction of male offspring is common and can lead to unnecessarily high costs (Ode and Heinz, 2002; Riccardo et al., 2018). Moreover, the reproduction of male offspring by heteronomous hyperparasitoids utilizes primary parasitoids, with each male offspring produced at the cost of a female parasitoid larva. This exacerbates the difficulties and costs of the rearing process. To control male production and increase female production, thereby enhancing the efficacy of biological control programs, it is essential to understand the factors influencing sex ratios.

Therefore, to address the controversy over sex ratio allocation in heteronomous hyperparasitoids and to determine if manipulating sex allocation can reduce interspecific competition and maximize the production of female parasitoids, we designed an experiment using an important heteronomous hyperparasitoid, *E. sophia*, a key biological control agent for the "super pest" *B. tabaci*. The experiment aims to: 1. Determine whether *E. sophia* can adjust the sex ratio of its offspring under different host resource conditions (varying host densities and host ratios). 2. If the females have the ability to regulate offspring sex ratios, identify the specific behaviors they employ to make these adjustments.

2 Materials and methods

2.1 Insect culture and host plant

The *B. tabaci* MEAM1 laboratory colony was obtained from the greenhouses at the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (CAAS) in Beijing. This population has been continuously grown under greenhouse conditions for 4 years, with annual supplementation from wild populations to rejuvenate it, and has never been exposed to pesticides. Laboratory colonies of *E. sophia* were generously provided by the Vegetable Integrated Pest Management Laboratory at the Texas Agricultural Experiment Station in Weslaco, TX, USA. To establish colonies of *E. sophia*, *B. tabaci* served as the host insect, and cotton plants (cv. xinke no.8, Hebei Zhongchuang Seed Technology Co. Ltd., China) were used as host plants in the laboratory experiments. Cotton plants, approximately 20 cm in height with 4–5 fully expanded leaves, were selected for this purpose. All host plants and insect colonies were meticulously maintained at Langfang Experimental Station (39°30’N, 116°36’E), Langfang, Hebei Province, China, under controlled conditions of 26±2°C, 65%±5% RH, and a 14 L: 10 D regime.

To prepare the culture medium, leaf discs containing primary hosts (third instar nymphs of *B. tabaci*) and secondary hosts (nymphs of *B. tabaci*, which had been

parasitized by *E. sophia* and the parasitoid larvae developed into the third instar to the pre-pupal stage), 50 pairs of whitefly adults were introduced into microscopic insect cages (specifications: 3 cm in diameter, 1 cm in height, covered with 120-mesh gauze) placed on cotton leaves. The adults were removed 24 hours after laying eggs. After 6 days, the same micro-insect cage was utilized to introduce 30 pairs of adult whiteflies, and the adults were removed 24 hours after egg-laying. Following the removal of the second batch of adults after 6-8 days, 15-20 mating *E. sophia* females were introduced into the micro-insect cage when the eggs laid by the first batch of whitefly adults developed into third instar nymphs. After 24 hours of egg-laying, the females were removed. A mesh bag (10 × 10 cm, 120-mesh gauze) was placed on the leaves to prevent contamination. After 6 days, appropriately aged primary and secondary hosts were distributed on the leaves. A 1% agar solution was poured into 2/3 of the Petri dish (d = 3.5 cm, S = 9.6 cm²), and circular shapes (d=3.5 cm, S = 9.6 cm²) of cotton leaves with primary and secondary hosts were cut. When the agar solution was about to solidify, small tweezers were used to place the leaves into the Petri dish, ensuring they adhered tightly to the agar for preservation. After the agar solidified, the leaf disc was examined under a dissecting microscope. According to the experimental requirements, a corresponding number of primary and secondary hosts were retained, and any excess nymphs and pupae of the whitefly that did not meet the experimental conditions were removed.

2.2 Offspring sex ratio of *Encarsia sophia* under varied host resources

Building upon our previous investigations into the host parasitization behavior of *E. sophia* females, we established three distinct parasitism scenarios characterized by varying host abundance in relation to the number of eggs the female wasp could lay: insufficient, moderate, and sufficient. The ratio of secondary hosts to the total number of hosts was set at 0.2, with host densities configured at 30, 50, and 70 hosts per dish (S = 9.6 cm²) (Sun, 2014). Specifically, for a host density of 30 hosts per dish (S = 9.6 cm²), the secondary host proportions were set to 0.5 and 0.2, and for a host density of 50 hosts per dish (S = 9.6 cm²), the secondary host proportions were set to 0.8 and 0.2.

Placing individual unmated males and single virgin females in Petri dishes (d = 3.5 cm, S = 9.6 cm²), we removed males once mating behavior was observed. Subsequently, mated females were introduced into culture dish leaf discs containing primary and secondary hosts. The diameter of the leaf disc was 3.5 cm, with an area of 9.6 cm². When investigating the impact of host density on offspring sex ratios, under a secondary host ratio of 0.2, host densities were set at 30, 50, and 70 individuals per leaf disc. When exploring the effect of host ratio on offspring sex ratios, at a host density of 30 individuals per leaf disc, the proportion of secondary hosts to the total host population was set at 0.5 and 0.2. Under a host density of 50 individuals per leaf disc, the proportion of secondary hosts to the total host population was set at 0.8 and 0.2. The culture dish leaf discs were covered with plastic wrap, pierced with insect pins, and the female wasps were transferred to leaf discs with the same oviposition environment every 24 hours for a continuous experiment over 5 days. After removing

the female wasps for 5 days, the number of parasitized primary and secondary hosts was recorded under a dissecting microscope, representing the number of eggs laid by female wasps for female and male offspring, respectively. The quantity of primary and secondary hosts consumed by the wasps was also recorded as the host feeding amount. Each treatment was repeated 20 times.

2.3 Host processing behavior of *Encarsia sophia* at different host resources

In Petri dishes (d = 3.5 cm, S = 9.6 cm²), individual unmated males and single virgin females were placed. Once mating behavior was observed, the males were promptly removed. Mated females were then introduced into culture dish leaf discs containing primary and secondary hosts (host densities set at 30, 50, 70 individuals per leaf disc, with a secondary host ratio of 0.2). The leaf discs used in the experiment were photographed and printed under a dissecting microscope, marking the positions and types of hosts and assigning each host a unique identifier. Upon entering the leaf discs, the females were covered with the lid of the culture dish. Under a dissecting microscope, the names, durations, and host identifiers for each behavior of the *E. sophia* females were recorded. After continuous observation for 1 hour, females were removed. Five days later, under the dissecting microscope, the number of parasitized primary and secondary hosts was recorded to determine whether *E. sophia* females laid eggs after ovipositor insertion into the hosts. This information was then matched with the previously assigned host identifiers, establishing the types of behaviors exhibited by females. Each treatment was repeated 20 times.

2.4 Data analysis

Data analysis was performed using SPSS 24.0 (SPSS Inc., Chicago, IL, USA). A two-way ANOVA test was employed to explore the factors influencing the offspring sex ratio, host parasitism and host feeding amount of *E. sophia* while maintaining a constant host ratio. Specifically, the analysis considered the effects of host density and female age. Similarly, under conditions where host density remained constant, a two-way ANOVA was used to investigate the impact of host ratio and female age on the offspring sex ratio and host parasitism. For assessing the significance of differences in normally distributed data, either in their original form or following transformation, an independent samples t-test was used for comparing two groups, and one-way ANOVA with Tukey's HSD was employed for comparing more than two groups. In cases where data, even after transformation, did not conform to normal distribution, the non-parametric Mann-Whitney test was used for comparing two groups, and the non-parametric Kruskal-Wallis test was employed for comparing more than two groups. In this study, the offspring sex ratio was defined as the proportion of male offspring relative to the total number of offspring, expressed as the ratio of offspring sex = number of male offspring / total number of offspring.

3 Results

3.1 Offspring sex ratio of *Encarsia sophia* under varied host resources

3.1.1 Effects of host resources on offspring sex ratio

The offspring sex ratio of *E. sophia* demonstrated significant responsiveness to variations in host density and the proportion of primary to secondary hosts. The interaction among these factors, along with female age (1-5 days old), exhibited no noteworthy impact on the offspring sex ratio of *E. sophia* (Supplementary Table S3-1). For instances where the secondary host proportion was 0.2 and host density of 30, 50, 70 hosts/9.6 cm², the daily average sex ratios of females were 0.19 ± 0.01, 0.31 ± 0.02, and 0.40 ± 0.02, respectively. A significant increase in sex ratio was observed with the rise in host density ($F_{2, 37}$ = 34.81, P < 0.001). Particularly, at host densities of 50 and 70 hosts/9.6 cm², the actual sex ratio was markedly higher than that observed at a host proportion of 0.2 (t=6.40, df=19, P < 0.001; t=10.52, df=19, P < 0.001) (Figure 3-1).

In a habitat with a host density of 30 hosts/9.6 cm², the sex ratios of offspring when the secondary host proportions were 0.5 and 0.2 were 0.47 ± 0.04 and 0.19 ± 0.01 respectively, which were not significantly different from the corresponding secondary host proportions(t = -0.80, df = 23, P = 0.432; t = -0.36, df = 19, P = 0.726), while there is a significant difference in offspring sex ratio between the two host proportions(t = 6.96, df = 30.02, P < 0.001) (Figure 3-2A). In a habitat with a host density of 50 hosts/9.6 cm², no significant difference was found in offspring ratio (0.76 ± 0.03) compared to the host ratio when the secondary host proportion was 0.8 (t = -1.37, df = 19, P = 0.186). Nevertheless, the offspring ratio (0.31 ± 0.02) was significantly higher when the secondary host ratio was 0.2 than the host proportion (t = 6.’à, df = 19, P < 0.001). Furthermore, the offspring ratio at a secondary host proportion of 0.8 was significantly higher than that at a secondary host proportion of 0.2 (t = 13.59, df = 38, P < 0.001) (Figure 3-2B).

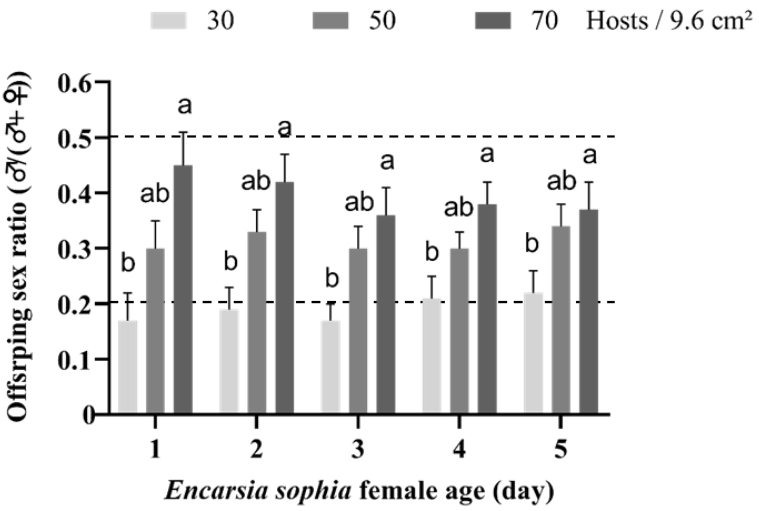


Figure 3-1. Effect of different host density and female age (1-5 days old) on offspring sex ratio of *Encarsia sophia* at a secondary host proportion of 0.2.

Note: Bar heads with different lowercase letters indicate significant differences (HSD test; $P < 0.05$) in sex ratio of offspring produced by female in the same age among different host density. The line at 0.2 represents the expected values matching the ratio of sex allocation to the ratio of secondary hosts, while the line at 0.5 indicates a trend toward a 1:1 sex allocation ratio.

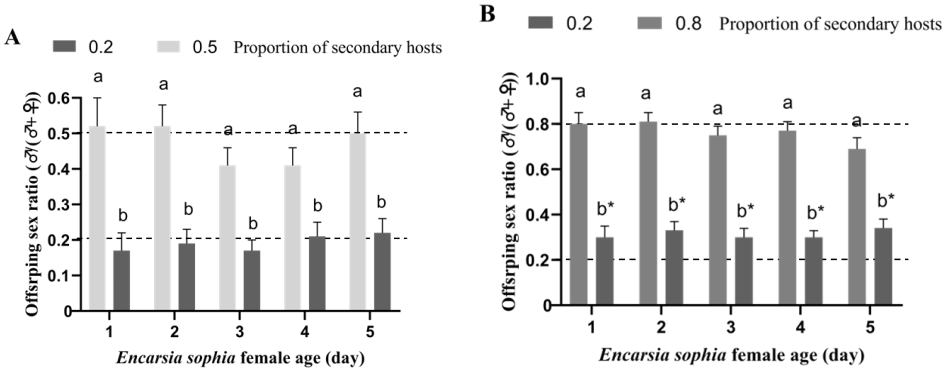


Figure 3-2. Effect of different secondary host ratio and female age (1-5 days old) on offspring sex ratio of *Encarsia sophia* under the conditions of host densities at 30 (A) and 50 (B) hosts/9.6 cm², respectively.

Note: The secondary host ratio is calculated as the number of secondary hosts divided by the total number of hosts. Bar labels with distinct lowercase letters denote

significant differences (t-test; $P < 0.05$) in the sex ratio of offspring produced by females of the same age across different host ratios.

* denotes a significant difference between the actual offspring sex ratio and the expected ratio based on the secondary host proportion (t-test; $P < 0.05$). The lines at 0.2, 0.5, and 0.8 represent the expected values matching the ratio of sex allocation to the ratio of secondary hosts.

3.1.2 Effects of host resources on the parasitism and host feeding

The variations in the ratio of primary hosts to secondary hosts and the age of females (1-5 days old) both have a significant impact on the parasitism level of females. However, the interaction between these two factors does not show a significant influence on parasitism. Similarly, changes in host density and the age of females (1-5 days old) significantly influence the parasitism level of females, with the interaction only significantly affecting the parasitism level on primary hosts. Moreover, alterations in host density significantly influence the feeding amount on hosts by females. The age of females (1-5 days old) and the interaction between this factor and host density do not have a significant impact on the feeding amount on hosts by females(Supplementary Table S3-2). At a secondary host proportion of 0.2 and host densities of 30, 50, and 70 hosts/9.6 cm², the overall parasitism by females on the two hosts exhibited no significant difference with the increasing host density ($F_{2, 57} = 3.16$, $P = 0.05$). However, parasitism on secondary hosts showed a significant increase ($F_{2, 57} = 15.41$, $P < 0.001$), while parasitism on primary hosts experienced a notable decrease ($F_{2, 57} = 8.49$, $P < 0.001$). The daily average host feeding behavior also significantly increased with the rise in host density (F2, 57 = 5.49, $P = 0.007$). Furthermore, at secondary host ratios of 0.5 and 0.8, compared to a secondary host ratio of 0.2 at the same host density, both total parasitism and overall parasitism significantly decreased (secondary host ratio 0.5, host density 30 hosts/9.6 cm²: t = -3.21, df = 25.112, $P = 0.004$; secondary host ratio 0.8, host density 50 hosts/9.6 cm²: t = -4.23, df = 29.775, $P < 0.001$) (Table 3-1).

Table 3-1. Effect of different host density or host ratio on daily mean number (± SE) of hosts parasitized, parasitized rate and host feeding by *Encarsia sophia* female evaluated in first 5 day after emergence.

Host density	Secondary host ratio	No. of hosts parasitized			No. of hosts parasitized rate %			No. of hosts fed
		Primary host	Secondary host	Primary host + secondary host	Primary host	Secondary host	Primary host +	Primary host

							secondary host	
30	0.2	6.4 ± 0.5 aA	1.5 ± 0.2 bB	7.9 ± 0.6 aA	26.7	25.0	26.3	4.5± 0.3 B
	0.5	3.2 ± 0.2 b	2.8 ± 0.2 a	6.0 ± 0.2 b	21.3	18.7	20.0	/
50	0.2	6.2 ± 0.5 aA	3.0 ± 0.3 bA	9.2 ± 0.6 aA	15.5	30.0	18.4	4.3± 0.3 B
	0.8	1.7 ± 0.2 b	4.9 ± 0.3 a	6.6 ± 0.4 b	17.0	12.3	13.2	/
70	0.2	5.1 ± 0.3 B	3.1 ± 0.2 A	8.2 ± 0.5 A	9.1	22.1	11.7	6.0± 0.5 A

Note: Different lowercase letters following data in the same column indicate significant differences under different host proportions at the same host density (t-test; $P < 0.05$), while different uppercase letters indicate significant differences under the same host proportions at different host densities (HSD test; $P < 0.05$).

3.2 Host processing behavior of *Encarsia sophia* at different host resources

3.2.1 Time allocation for host processing behavior

Under the condition that the proportion of secondary host is 0.2 and the host density is 30, 50 and 70 hosts/9.6 cm² respectively, the proportion of each processing behavior of *E. sophia* female to the primary host and the secondary host in the total time within 1 hour is calculated. With the increase of host density, the proportion of host search time in the total time decreased, the proportion of secondary host processing time (examination and laying) in the total time increased, and the proportion of primary host feeding in the total time increased. Therefore, *E. sophia* females perceived the change of host density within 1 hour after entering the habitat. Among them, under the condition that the host density is 70 hosts/9.6 cm², the search time for hosts was significantly less than 30, 50 hosts/9.6 cm² ($F_{2,49}$=5.387, P=0.008); The feeding time accounts for the proportion of the total time was significantly higher than the host density of 30 hosts/9.6 cm² ($F_{2,58}$ = 3.518, P=0.036)(Figure 3-3).

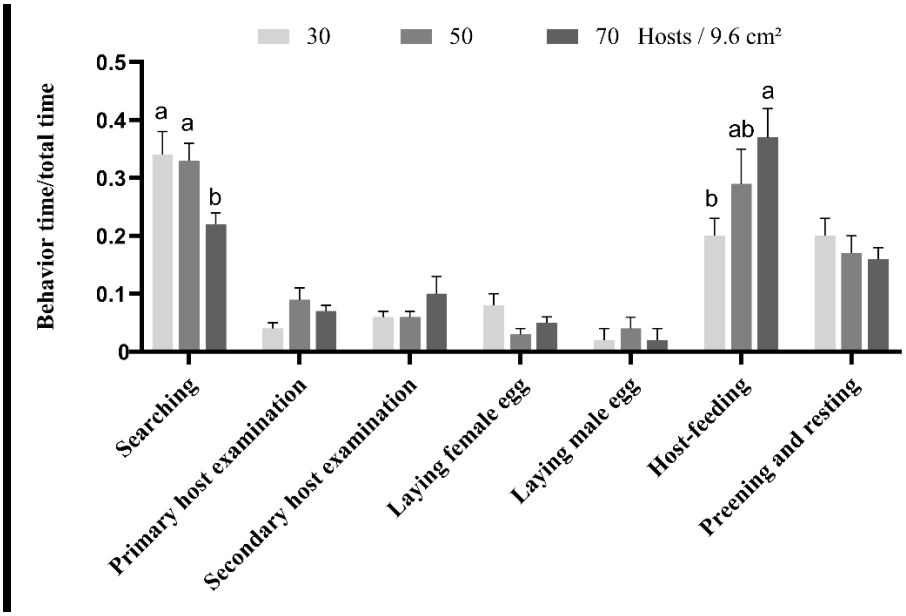


Figure 3-3. Relative time (in %) spent by *Encarsia sophia* female on host treating under the condition of different host density (the proportion of secondary host was 0.2).

Note: Bar heads with different lowercase letters indicate significant differences (HSD test; $P < 0.05$) in the rate of behavior time among different host density.

3.2.2 Encounter rate of secondary hosts

When females examine host in external, it was regarded as encounting the host. The proportion of secondary hosts provided in the experiment was 0.2, so the theoretical probability of encountering secondary hosts in the habitat (amount of encountering secondary hosts/total amount of encountering hosts) is 0.2. While the actual observation shows that the actual probability of encountering secondary hosts under different host densities (30, 50 and 70 hosts/9.6 cm²) is 0.36, 0.55 and 0.46 respectively, which are significantly higher than the theoretical value (t=3.34, df=19, *P*=0.003; t=6.00, df=22, *P*<0.001; t=4.68, df=20, *P*<0.001)(Table 3-2).

Table 3-2. Encounter rate of secondary host for *Encarsia sophia* with different host density (Mean ± SE; the proportion of primary host and secondary host was 0.2).

	Theory probability	Realistic probability		
		Host density 30	Host density 50	Host density 70
No. of second hosts/No. of hosts	0.20c	0.36 ± 0.05 b	0.55 ± 0.06 a	0.46 ± 0.06 ab

Note: Data followed by different lowercase letters indicate significantly different at 0.05 level (HSD text).

3.2.3 The number of females treated and accepted to the host

Females of *E. sophia* enter the habitat and locate the host through searching. Once the host is externally examined, it is considered as the initiation of host-treatment behavior. After examining the host, females make the choice of either accepting or rejecting it. In the case of an accepted primary host, females will either parasitize or feed, while for the accepted secondary host, females will hyperparasitize; however, feeding on secondary hosts is rarely observed. Based on statistical analysis, the proportion of females encountering the secondary host was 0.2, and the host density was set at 30, 50, and 70 hosts/9.6 cm², respectively. There was no significant difference in the treatment amounts for primary hosts, secondary hosts, and total hosts with the increase in host density (primary host: χ^2 =0.74, df =2, *P* =0.929; secondary host: χ^2 = 1.89, df =2, *P* =0.389; total: χ^2 = 0.81, df =2, *P* =0.667) (Figure 3-4);

There was no significant difference in the acceptance of the primary host, secondary host, and total host with the increase in host density (primary host: χ^2=1.28, df =2, *P* =0.526; secondary host: χ^2=1.63, df =2, *P* =0.443; total: χ^2=0.73, df =2, *P* =0.696). However, there was an adjustment in oviposition and feeding behavior after receiving the primary host. With the increase in host density, females shifted more of their egg-laying behaviors towards host-feeding behaviors ($F_{2,61}$=3.80, *P* =0.028) (Figure 3-5).

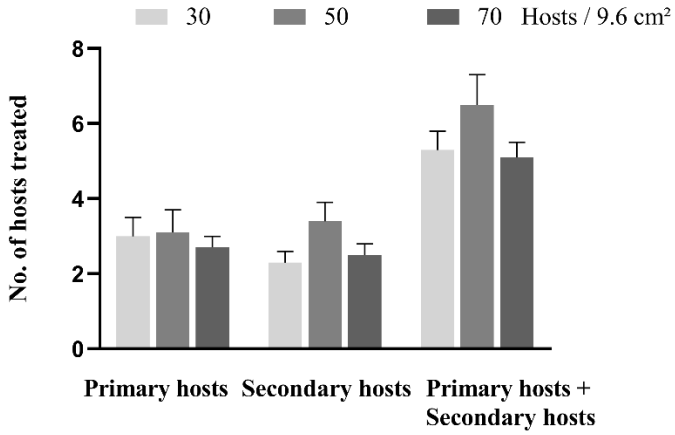


Figure 3-4. Abundance (Mean ± SE) of hosts treated by *Encarsia sophia* female in 1 h under the condition of different host density (with a secondary host proportion of 0.2).

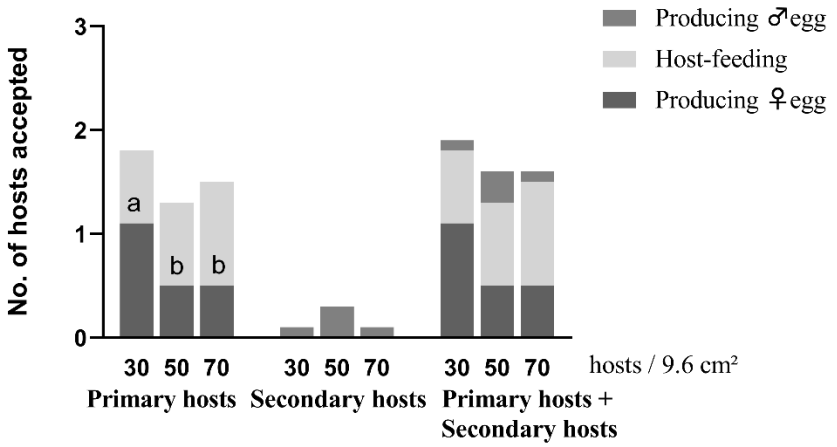


Figure 3-5. Abundance of hosts accepted by *Encarsia sophia* female in 1 h under the condition of different host density (with a secondary host proportion of 0.2).

Note: Bar heads with different lowercase letters indicate significant differences (HSD test; $P < 0.05$) in the number of female eggs among different host density.

4 Discussion

As an important biological control agent, the sex allocation of heteronomous hyperparasitoids remains a subject of debate. Here, we focus on *E. sophia*, the dominant parasitoid of *B. tabaci*. Our results, diverging from both Godfray et al.'s findings and those of Walter and Donaldson, indicated that under conditions where the ratio of secondary hosts exceeds that of primary hosts or in situations of low host density (host limitation), the offspring sex ratio of *E. sophia* adjusts based on the relative abundance of primary and secondary hosts. However, when the ratio of secondary hosts is low (<0.5), with an increase in host density, the offspring sex ratio tends toward 1:1. Godfray and colleagues proposed that the sex ratio of heteronomous hyperparasitoid tends toward 1:1 with increasing host density, independent of the relative abundance of primary and secondary hosts. However, their findings did not encompass situations with different host ratios at the same host density, limiting the applicability to diverse host scenarii for heteronomous hyperparasitoid (Godfray and Waage, 1990; Godfray and Hunter, 1992, 1994). Also, Donaldson and Walter's results suggested that the sex ratio of heteronomous hyperparasitoids is solely influenced by the relative abundance of primary and secondary hosts (Walter and Donaldson, 1994). Yet, this can be attributed to an experimental design focusing exclusively on a host density of 50, lacking consideration for other host densities. Therefore, drawing conclusions that the sex ratio of heteronomous hyperparasitoid offspring is entirely or unrelated to host ratio based on existing results may be prematured.

The sex ratio of insects denotes the proportion of female and male phenotypes within the insect population during a specific period (Abe et al., 2021). Consequently, the sex ratio of parasitoids is most directly reflected in the ratio of female to male offspring, representing the proportion of females producing female and male eggs. Heteronomous hyperparasitoids, owing to their robust host feeding ability, primarily feed on primary hosts and infrequently on secondary hosts (Yang et al., 2012). This feeding behavior has an impact on the relative abundance of primary and secondary hosts. To elucidate the sex ratio regulation mechanism of heteronomous hyperparasitoids, we investigated the parasitism and feeding behavior of females under varying primary and secondary host resources. The results contribute to the sex ratio theory outlined above. Under conditions of low host density (insufficient number of hosts), the parasitism rate on primary and secondary hosts is equivalent, and the sex ratio of female offspring is determined by the relative abundance of the two hosts. This finding aligns with previous studies by Kuenzel (1975), Williams (1977), and Hunter (1989), confirming a significant positive correlation between the number of male offspring of *Encarsia pergandiella* and the proportion of suitable-age secondary hosts based on field and laboratory data (Kuenzel, 1975; Williams, 1977; Hunter, 1989). However, through statistical analysis of the parasitism and feeding levels of *E. sophia* under different host conditions, we observed that with an increase in host density (sufficient number of hosts) and a low proportion of secondary hosts, the parasitism rate on secondary hosts significantly surpassed that on primary hosts. Consequently, there was a decrease in female production and an increase in male production. Simultaneously, there was an escalation in feeding on primary hosts, resulting in a reduction in the number of eggs laid by female offspring and an increase in the number of eggs laid by male offspring. Therefore, the sex ratio of offspring tended to be 1:1. Furthermore, under the same host density, an increase in the proportion of secondary hosts results in a significant decrease in both the total parasitism quantity and parasitism rate of *E. sophia* females. This suggests that an environment with excessively high proportions of secondary hosts negatively influences the parasitism rate of the parasitoid, potentially linked to the parasitoid's preference for different host types. Subsequent experiments could explore the dwell time of the parasitoid in various environments, confirming whether females are inclined to reduce their stay when exposed to environments with excessively high proportions of secondary hosts, thereby leading to a potential early departure. In essence, this behavior may mitigate the occurrence of intense interspecific competition among heteronomous hyperparasitoids.

Through the aforementioned results, we have established that heteronomous hyperparasitoids can maintain the stability of offspring sex ratios by regulating female egg production, male egg production, and feeding behaviors. This leads us to the next question: how does the hyperparasitoid adjust female production, male production, and host feeding through behavioral changes upon perceiving host density, ultimately influencing the sex ratio of offspring? By observing the host-handling behavior of E. sophia females after entering the habitat, we found that females can perceive host density within one hour of entering the habitat. The findings indicated that females

can perceive host density within one hour after entering the habitat. Host processing behaviors under the three host densities exhibited significant temporal variations. With the escalation of host density, the proportion of time allocated to host searching decreased, while the proportion of processing time (examination and oviposition) on secondary hosts and feeding time on primary hosts significantly increased. Additionally, Hunter (1993) suggested that the sex ratio of *E. pergandiella* might be linked to the proportion of females encountering secondary hosts, and Avilla (1987) proposed that differences in parasitism between primary and secondary hosts may be attributed to variations in the encounter rate and treatment time of hyperparasitoids toward the two hosts. Analyzing the encounter rate of *E. sophia* females with secondary hosts under different host densities, we observed an increase in the encounter rate with host density, surpassing the theoretical probability. Hence, the likelihood of females encountering secondary hosts was higher, aligning with Avilla's observations on *Encarsia tricolor* and Hunter's findings with *E. pergandiella*. Females exhibit a preference for secondary hosts in both primary and secondary hosts (Avilla and Copland, 1987; Hunter, 1993).

Contrastingly, when comparing the encounter rate of secondary hosts under the three host densities with the corresponding offspring sex ratio, it was noted that the encounter rate of secondary hosts was higher than the corresponding offspring sex ratio. This implies that the offspring sex ratio of hyperparasitoids is not solely determined by the encounter rate of secondary hosts. Hunter proposed that if females are more prone to accepting or rejecting a host frequently, the offspring sex ratio of parasitoids may not directly reflect the proportion of primary hosts to secondary hosts in the habitat (Hunter, 1989). We further analyzed the number of *E. sophia* females treating and accepting two hosts under three host densities. The results showed no significant difference in the number of primary hosts, secondary hosts, and the total number of hosts, which contradicts the observed sex ratio of *E. sophia*'s offspring. Consequently, we delved into understanding how *E. sophia* makes behavioral choices between primary and secondary hosts, ultimately influencing the number of male and female offspring. Upon further analysis, it was discovered that although the number of females treating and accepting primary hosts did not significantly differ with changing host density, the treatment behavior of primary hosts changed with increasing host density. Partial oviposition behaviors on primary hosts transformed into feeding behaviors. Consequently, when the number of secondary hosts is low, the number of female offspring is reduced, leading to a trend toward an equal ratio of male and female offspring. Hunter's sex allocation study of *E. pergandiella* revealed that the female oviposition sex ratio was affected by the proportion of secondary hosts, but to a lesser extent than predicted solely from the proportion of secondary hosts. This discrepancy may be attributed to the oversight of female feeding behavior on primary hosts (Hunter, 1993).

Furthermore, research on the sex ratio adjustment capability of heteronomous hyperparasitoids not only enriches the theoretical understanding of the sex ratio in this special type of parasitoid wasps but also provides a reference for evaluating their application and large-scale rearing. Our study demonstrates that heteronomous

hyperparasitoids can adjust the sex ratio of their offspring under different host resource conditions, which is crucial for population stability. Huang and Warsi have suggested that adjusting the parasitoid-to-host ratio can reduce the population fluctuation range of parasitoids and increase their survival probability (Huang et al., 2016; Warsi et al., 2023). This could be one reason why such parasitoids become dominant populations in the wild, making them excellent candidates for biological control (Yang et al., 2022; Tize et al., 2023). However, in large-scale rearing, the high male-to-female ratio due to parthenogenesis and hyperparasitism characteristics of these parasitoids poses challenges (Katono et al., 2023). Maximizing female production and ensuring the establishment of released parasitoids are fundamental to optimizing biological control programs (Riccardo et al., 2018; Hougardy et al., 2022). Our results indicate that for heteronomous hyperparasitoids, higher host density does not necessarily lead to more female offspring. At a secondary host proportion of 0.2 and a host density of 30 hosts/9.6 cm^2, i.e., under host limitation with ample parasitoid eggs, the maximum number of female offspring was obtained with minimal consumption of secondary hosts (primary parasitoid larvae). This condition minimizes costs and maximizes yield, representing the optimal rearing strategy. We can use this information to calculate the ratio of maternal parasitoids to primary and secondary hosts, achieving large-scale production of female offspring.

5. Supplementary data

Table S3-1. The results of ANOVA for offspring sex ratio of *Encarsia sophia* affected by host resources and female age.

Source	df	Mean Square	F	*P*
Secondary host ratio is 0.2, with host densities of 30, 50, and 70 hosts / 9.6 cm².				
Host density	2	1.000	26.19	< 0.001*
Age(day)	4	0.012	0.32	0.861
Host density × age(day)	8	0.014	0.36	0.942
Error	278	0.038		
Total	293			
Host density is 30 hosts /9.6 cm², with secondary host ratios of 0.5 and 0.2.				
Host ratio	1	4.101	62.32	< 0.001*
Age(day)	4	0.038	0.58	0.680
Host ratio × age(day)	4	0.040	0.61	0.660
Error	207	0.066		
Total	217			
Host density is 50 hosts /9.6 cm², with secondary host ratios of 0.8 and 0.2.				
Host ratio	1	9.780	284.59	< 0.001*
Age(day)	4	0.019	0.55	0.696
Host ratio × age(day)	4	0.035	1.02	0.400
Error	187	0.034		
Total	197			

Note:*Indicates that the interaction between host density/host ratio and the age of the female significantly affects the offspring sex ratio.

Table S3-2. The results of ANOVA for number of hosts parasitized and host fed by *Encarsia sophia* female affected by host density and female age or host ratio and female age.

Source	df	Mean Square	F	*P*
Host density is 30 hosts /9.6 cm², with secondary host ratios of 0.5 and 0.2.				
Number of secondary hosts parasitized				
Host ratio	1	80.281	31.68	< 0.001*
Age(day)	4	12.500	4.93	0.001*
Host ratio × age(day)	4	0.674	0.27	0.900
Error	209	2.534		
Total	219			
Number of primary hosts parasitized				
Host ratio	1	558.986	87.45	< 0.001*
Age(day)	4	73.456	11.49	< 0.001*
Host ratio × age(day)	4	2.565	0.40	0.808
Error	209	6.392		
Total	219			
Number of primary hosts and secondary hosts parasitized				
Host ratio	1	215.587	28.37	< 0.001*
Age(day)	4	137.98	18.15	< 0.001*
Host ratio × age(day)	4	2.75	0.36	0.836
Error	209	7.60		
Total	219			
Host density is 50 hosts /9.6 cm², with secondary host ratios of 0.8 and 0.2.				
Number of secondary hosts parasitized				
Host ratio	1	170.966	45.11	< 0.001*
Age(day)	4	38.444	10.14	< 0.001*
Host ratio × age(day)	4	4.963	1.31	0.268
Error	187	3.790		
Total	197			
Number of primary hosts parasitized				
Host ratio	1	1354.256	224.41	< 0.001*
Age(day)	4	51.991	8.62	< 0.001*
Host ratio × age(day)	4	17.233	2.86	0.025*

Error	187	6.035		
Total	197			
Number of primary hosts and secondary hosts parasitized				
Host ratio	1	562.866	62.94	< 0.001*
Age(day)	4	172.611	19.30	< 0.001*
Host ratio × age(day)	4	10.634	1.19	0.317
Error	187	8.942		
Total	197			
Secondary host ratio is 0.2, with host densities of 30, 50, and 70 hosts / 9.6 cm².				
Number of secondary hosts parasitized				
Host density	2	74.696	26.88	< 0.001*
Age(day)	4	19.933	7.17	< 0.001*
Host density × age(day)	8	0.760	0.27	0.974
Error	279	2.779		
Total	294			
Number of primary hosts parasitized				
Host density	2	91.212	10.53	< 0.001*
Age(day)	4	127.623	14.74	< 0.001*
Host density × age(day)	8	3.487	0.403	0.919
Error	279	8.660		
Total	294			
Number of primary hosts and secondary hosts parasitized				
Host density	2	125.478	11.74	< 0.001*
Age(day)	4	240.038	22.45	< 0.001*
Host density × age(day)	8	6.308	0.59	0.786
Error	279	10.692		
Total	294			
Number of hosts fed				
Host density	2	81.68	10.15	< 0.001*
Age (dya)	4	16.608	2.06	0.086
Host density × age	8	2.363	0.29	0.968
Error	281	8.05		
Total	296			

Note:*Indicates that the interaction between host density/host ratio and the age of the female significantly affects the parasitism/feeding rate of the female.

In **Chapter 3**, the sex ratio adjustment ability of *Encarsia sophia* under different host resource conditions was explored. The study demonstrated that *E. sophia* can flexibly adjust the sex ratio of its offspring based on the relative abundance and density of primary and secondary hosts, optimizing its reproductive fitness. This research provides crucial insights into how heteronomous hyperparasitoids adapt to complex ecological environments through sex ratio regulation, highlighting its adaptive behavioral strategies. However, the molecular mechanisms underlying this sex ratio adjustment remain unclear. Naturally progressing from this, **Chapter 4** delves deeper into the issue, focusing on the genome sequencing of *E. sophia* in an effort to uncover the genetic basis behind its complex behavioral decisions and lay the groundwork for investigating its molecular recognition mechanisms.

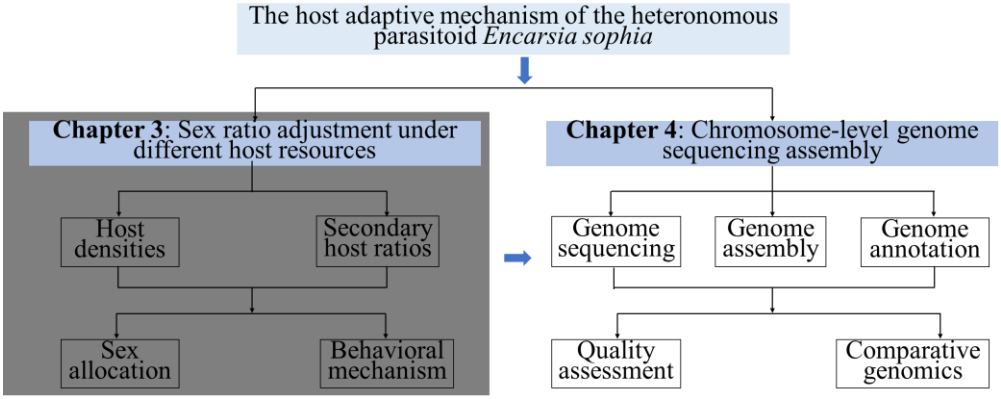


Figure 3-6. The transition from Chapter 3 to Chapter 4 in the project "The host adaptive mechanism of the heteronomous parasitoid *Encarsia sophia*".

Chapter 4

A chromosome-level genome assembly of the heteronomous hyperparasitoid wasp *Encarsia sophia*

"*Biological control is not about eradicating pests but achieving a balance where natural enemies keep them in check.*"

Richard Greathead

Adapted from:

Man, X., Huang, C., Wu, S., Guo, J., Wan, F., Francis, F., Yang, N., Liu, W. A chromosome-level genome assembly of the heteronomous hyperparasitoid wasp *Encarsia sophia*. *Scientific Data*. 11, 1250 (2024). https://doi.org/10.1038/s41597-024-04040-2

Abstract

Encarsia sophia, a heteronomous hyperparasitoid wasp, is a well-known biological control agent of pests. However, genomic information remains lacking for further fundamental molecular investigations and multitrophic interaction understanding. In this study, we present the chromosome-level genome assembly of *E. sophia*, providing key insights into its genomics. Findings: Here, we present a chromosome-level genome assembly for *E. sophia*, utilizing Illumina, PacBio HiFi, and Hi-C technologies. The assembled genome size is 398.3 Mb, featuring a contig N50 of 1.0 Mb and a scaffold N50 of 74.0 Mb. The BUSCO completeness score is 97.1%, and the genome coverage reaches 99.1%. Leveraging Hi-C assisted assembly, the genome was successfully organized into five chromosomes, achieving a mounting rate of 95.1%. Repetitive sequences constitute 54.6% of the genome, and a total of 14,914 protein-coding genes were predicted, with 95.5% of them functionally annotated. Conclusions: The high-quality genome assembly of *E. sophia* is a groundbreaking achievement, marking the first complete genome for a heteronomous hyperparasitoid wasp. This genomic milestone provides valuable insights into the complex evolution and host interactions specific to heteronomous hyperparasitoids, laying the foundation for extensive research in biological control.

***Keywords*:** *Encarsia sophia*; heteronomous hyperparasitoid; genome assembly; comparative genomes

1. Background & Summary

The Hymenoptera, one of the four largest orders in the class Insecta, is one of the most species-rich groups of insects. With the advancement of sequencing technologies, this order has become a hotspot in insect genomics research (Ye et al., 2020; Zhong et al., 2023). Currently, the number of sequenced Hymenoptera genomes has reached 557 (on April 2024, based on statistics from NCBI), with 388 species sequenced in the past three years, and annotation information submitted for 125 species. Among these sequenced Hymenoptera species, 258 belong to parasitoids, primarily including 36 species of Cynipoidea, 75 species of Chalcidoidea, 98 species of Ichneumonoidea, 42 species of Proctotrupoidea, 6 species of Chrysidoidea, and 1 species of Orussidea.

Encarsia sophia (Hymenoptera: Aphelinidae) is a dominant parasitoid of the "super pest" *Bemisia tabaci* (Hemiptera: Aleyrodidae), serving as a crucial biological control agent against global populations of whiteflies due to its remarkable parasitic and destructive capabilities on the host (Katono et al., 2022; Caspary et al., 2023; Charles, 2024). The reproductive strategy of this parasitoid is rather unique, being a typical heteronomous hyperparasitoid. Males and females develop heteronomously, obtaining their nutritional resources from different host insects. Females, the primary parasitoids, arise from fertilized eggs and parasitize directly within the target host insect, feeding on the larvae or nymphs of the host to complete their development. Conversely, males, arising from unfertilized eggs, act as hyperparasitoids and can only parasitize secondary hosts, i.e., those already parasitized by the primary parasitoids, feeding on the larvae of the primary parasitoids to complete their development(Walter, 1983; Mills and Gutierrez, 1996; Williams, 1996; Hunter and Woolley, 2001). Here, mated female *E. sophia* parasitize directly within the nymphs of the *B. tabaci*, laying fertilized eggs that develop into female offspring, serving as primary parasitoids. Unmated females, on the other hand, can only parasitize secondary hosts, laying unfertilized eggs within the nymphs of conspecific or heterospecific parasitoids already parasitized within the whitefly nymphs, producing male offspring, acting as hyperparasitoids(Yang et al., 2012; Xu et al., 2013) So far, no genome of a heteronomous parasitoid has been reported. In order to gain deeper insights into the characteristics of such parasitoids, we conducted whole-genome sequencing and chromosomal-level assembly of *E. sophia* using Illumina, PacBio, and Hi-C technologies. We also annotated protein-coding genes and analyzed the evolution of gene families across different parasitoid species.

2. Methods

2.1 Parasitoid Wasp Collection and Sequencing

Encarsia sophia population, introduced in 2008 from the Vegetable Pest Integrated Management Laboratory at Texas A&M University, USA. They were reared in the insectarium of the Laboratory of Biological Invasion Research at the Langfang Research and Development Base of the Chinese Academy of Agricultural Sciences, using cotton plant *B. tabaci* nymphs as hosts (26±1°C, RH65±5%, light cycle 14L:10D). The *B. tabaci* laboratory population originates from the MEAM1

population maintained by the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (CAAS), in a greenhouse at the Institute of Plant Protection, CAAS, with no history of pesticide use. The cotton variety used is CCRI 49. *E sophia* is a typical heteronomous hyperparasitoid with a unique reproductive strategy: females act as primary parasitoids, parasitizing first- to fourth-instar *B. tabaci* nymphs (primary hosts). In contrast, solitary females produce male offspring, acting as secondary parasitoids parasitizing conspecific or heterospecific parasitoid larvae inside *B. tabaci* nymphs (secondary hosts). Given that males are secondary parasitoids, we collected newly emerged females for sequencing. To obtain newly emerged parasitoids, we used insect pins to transfer females from black pupae to centrifuge tubes (1.5 mL). We checked daily for newly emerged adults, collecting a total of 4,000 female adults for DNA extraction, using the QIAamp DNA Mini Kit (QIAGEN). After extraction, DNA purity, concentration, and integrity were assessed using NanoDrop 2000&8000, Qubit Fluorometer, and Agilent 4200 Bioanalyzer, respectively.

2.2 Genome size estimation and assembly

The qualified DNA samples from *E. sophia* were randomly fragmented using a Covaris ultrasonic disruptor followed by further processes such as end repair, A-tailing, adapter ligation, purification, PCR amplification, and other steps to complete the entire library preparation. The constructed library was subjected to paired-end sequencing using Illumina HiSeq. Based on the filtered clean reads, a survey analysis was conducted using kmer17 (Kingsford, 2011). yielding the following estimations: a genome size of 412.21 Mbp, corrected to 404.2 Mbp, heterozygosity rate of 0.52%, and a repeat sequence proportion of 52.84% (Table 4-1). The sequencing data were assembled using Soapdenovo software, followed by assembly into scaffolds using kmer41. The contig N50 was determined to be 1,272 bp with a total length of 318,591,742 bp, while the scaffold N50 was 2,192 bp with a total length of 328,391,604 bp (Table 4-2).

Sequencing was conducted using the PacBio platform, resulting in a total sequencing volume of 148G with a coverage depth of 366.16X (calculated based on the survey-estimated genome size of 404.20M). Additionally, an Illumina short-insert library was constructed and sequenced on the Illumina platform (Table 4-3). Using the sequencing data, de novo assembly of the *E. sophia* genome was performed with HiFiasm (Cheng et al., 2021). The genome contig N50 reached 1.33Mbp, and the scaffold N50 also reached 1.33Mbp (sequences above 100bp were selected for the assembly results) (Table 4-4).

To obtain the chromosome-level genome of *E. sophia*, a Hi-C sequencing library was constructed using Hi-C technology (Belaghzal et al., 2017). incorporating DNA from 20,000 female adults. Hi-C data were obtained from the sequencing, and the contigs/scaffolds assembled were anchored to approximate chromosome-level using the All-hic software (Zhang et al., 2019). Subsequently, the juicebox software (https://github.com/aidenlab/Juicebox) was utilized for manual correction based on chromosomal interaction intensity, resulting in the final chromosome-level genome

of *E. sophia* (Table 4-5). Following Hi-C-assisted assembly, the *E. sophia* genome assembled at the chromosome level comprises a total of 5 sequences, with an additional 189 sequences remaining unassembled at the chromosome level. The contig total length is 398,185,814 bp, and the contig N50 length reaches 715,578 bp. The scaffold total length is 398,274,414 bp, and the scaffold N50 length is 73,963,014 bp. A heatmap was generated to illustrate the interactions of each chromosome (Figure 4-1). The genome mapping rate achieved is 95.1% (Table 4-6,7). (Results were based on contigs above 100bp for assembly statistics).

Table 4-1. *Encarsia sophia* genome feature statistics obtained by Kmer analysis.

Sample	***Encarsia sophia***
Kmer	17
Depth	87
n_kmer	35,862,604,357
Genome_size(M)	412.21
Revised Genome_size(M)	404.2
Heterozygous_rate(%)	0.52
Repeat_rate(%)	52.84

Table 4-2. *Encarsia sophia* genome assembly to scaffold results.

	Total_length	**Total_number**	**Max_length**	**N50_length**	**N90_length**
Contig	**318,591,742**	**699,645**	**91,064**	**1,272**	**133**
Scaffold	328,391,604	601,156	178,874	2,192	146

Table 4-3. Statistics of the DNA/RNA sequence data used for genome assembly.

Library	**Insert size(bp)**	**Total data (G)**	**Read length (bp)**	**Sequence coverage (X)**
Illumina	350	49.70	150	122.96
PacBio	-	148	-	366.16
Hi-C	350	2.37	150	98.54

Table 4-4. *Encarsia sophia* genome denovo assembly results statistics.

	Total_length	**Total_number**	**Max_length**	**N50_length**	**N90_length**
Contig	338,576,684	1,144	295,958	1,327,545	136,066
Scaffold	328,391,604	1,144	295,958	1,327,545	136,066

Table 4-5. *Encarsia sophia* genome assembly results statistics of de novo and after Hi-C scaffolding.

Sample	Contig length	Scaffold length	Contig number	Scaffold number
Total	398,185,814	398,274,414	1,080	194
Max	4,052,312	163,268,332	-	-
Number>=2000	-	-	1080	194
N50	715,578	73,963,014	161	2
N60	558,990	72,460,500	224	3
N70	435,605	72,460,500	304	3
N80	326,172	38,401,749	410	4
N90	185,480	30,794,298	570	5

Table 4-6. *Encarsia sophia* single chromosome cluster number and length statistics of Hi-C assemble.

Sequeues ID	Cluster number	Sequeues length
Chr1	349	163,268,332
Chr2	164	73,963,014
Chr3	157	72,460,500
Chr4	142	38,401,749
Chr5	79	30,794,298

Table 4-7. *Encarsia sophia* genome mapping rate of de novo and afer Hi-C scaffolding.

Class	Scaffold number	Total length
Place	5	378,887,893
Unplace	189	19,386,521
Total	194	398,274,414
Mapping rate	95.13%	

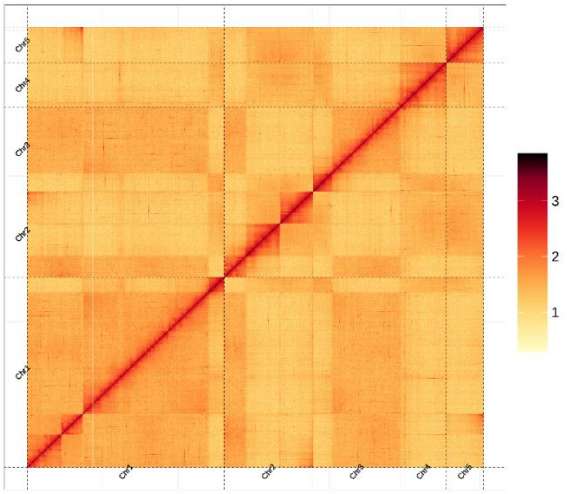


Figure 4-1. Genome-wide all by all Hi-C interaction heatmap of *Encarsia sophia* (5 chromosomes, resolution100 kb). The intensity of chromosomal interactions is shown on the right shading gradient. Intrachromosomal interactions (red blocks in the diagonal) are much stronger than interchromosomal interactions (light yellow blocks).

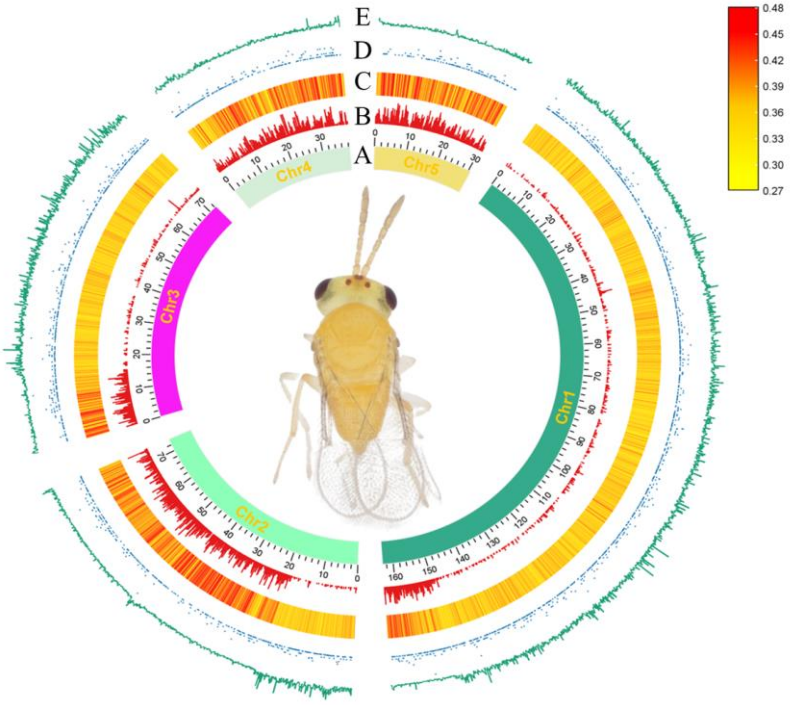


Figure 4-2. Chromosome-level genome assembly results information circle plot. A: chromosome information, B: gene density, C: GC content, D: ncRNA density, E: repeat density.

2.3 Genome quality assessment

We employed different methods to assess the sequence integrity, consistency, and accuracy of the genome assembly. Firstly, the integrity of *E. sophia* genome assembly was evaluated using BUSCO assessment (Manni et al., 2021) with software such as metaeuk and hmmer. The assembly resulted in 97.1% complete BUSCO genes, with 92.1% being single-copy genes and 5.0% being completely duplicated genes. Additionally, a core gene library comprising 248 conservative genes present in six eukaryotic model organisms was used for CEGMA assessment (Parra et al., 2007) using tblastn, genewise, and geneid software. The assembly successfully identified 233 out of 248 core eukaryotic genes, indicating a completeness rate of 93.9%. Secondly, the sequence consistency of the *E. sophia* genome was assessed by aligning short-insert library reads using BWA software (http://bio-bwa.sourceforge.net/) (Langmead and Salzberg, 2012). The analysis revealed a read alignment rate of approximately 97.6% and a genome coverage rate of around 99.1%, demonstrating strong consistency between the reads and the assembled genome. SNP calling was performed using samtools (http://samtools.sourceforge.net/) on the BWA alignment results, and after filtering and statistical analysis (Li, 2011), the genome exhibited a heterozygous SNP rate of 0.317095% and a homozygous SNP rate of 0.000943%, indicating a high single-base accuracy in the assembly. Thirdly, the sequence accuracy of the *E. sophia* genome was assessed using Merqury software (https://github.com/marbl/merqury) with Illumina sequencing data. The quality value (Qv) of the genome, calculated based on K-mer using the Merqury-mash module (Koren et al., 2017; Rhie et al., 2020), was determined to be 33.6653, indicating a base accuracy rate exceeding 99.9%. In conclusion, the *E. sophia* genome assembly exhibits good consistency, completeness, and accuracy (Table 4-8).

Table 4-8. *Encarsia sophia* genome assembly quality assessment results.

Evaluation indicators	results
BUSCO	C:97.1%[S:92.1%,D:5.0%],F:0.6%,M:2.3%,n:1367
CEGMA	93.95 %Completeness
Reads	97.58% Mapping rate;99.10% Coverage
SNP	0.317095% Heterozygosis;0.000943% Homology
Qv	33.6653

2.4 Genome annotation

Our repetitive annotation method employs a comprehensive strategy based on homology alignment and de novo search to identify repetitive sequences throughout the entire genome. We utilized TRF (http://tandem.bu.edu/trf/trf.html) (Benson, 1999) for ab initio prediction, extracting tandem repeat sequences. Homology prediction involved the use of the common Repbase database (http://www.girinst.org/repbase) (Bao, 2015), applying RepeatMasker (http://www.repeatmasker.org/) (Tarailo-Graovac and Chen, 2009) software and its internal script (RepeatProteinMask) to extract repetitive regions with default parameters. For de novo prediction, we

employed LTR_FINDER (http://tlife.fudan.edu.cn/ltr_finder/) (Xu and Wang, 2007), RepeatScout (http://www.repeatmasker.org/), and RepeatModeler (http://www.repeatmasker.org/RepeatModeler.html) (Flynn et al., 2020) to establish a de novo repetitive element database. Subsequently, all repetitive sequences with lengths greater than 100bp and a 'N' ratio less than 5% constituted the raw transposable element (TE) library. A custom library, formed by combining Repbase and our de novo TE library and processed through uclust to create a non-redundant library, was provided to RepeatMasker for DNA-level repetitive sequence identification. The *Encarsia sophia* genome contains 214.7 Mb of repetitive sequences, constituting 53.92% of the genome. Among them, long terminal repeats (LTRs) are the most abundant, accounting for 34.59% of the total, followed by Unknown (12.17%), 7.18% DNA elements, 3.96% long interspersed nuclear elements (LINEs), and only 0.02% short interspersed nuclear elements (SINEs) (Table 4-9).

The annotation of protein-coding genes in *E. sophia* genome combines de novo prediction, homology-based prediction, and RNA-Seq-assisted prediction for gene modelling (Mei et al., 2022). For de novo gene prediction, our automated gene prediction pipeline utilized Augustus (v3.2.3) (http://bioinf.uni-greifswald.de/augustus/) (Stanke et al., 2006), Geneid (v1.4), Genescan (v1.0), GlimmerHMM (v3.04) (http://ccb.jhu.edu/software/glimmerhmm/) (Majoros et al., 2004), and SNAP (http://homepage.mac.com/iankorf/) (Korf, 2004). Homologous protein sequences were downloaded from NCBI *Nasonia vitripennis* (Nvit), *Ceratosolen solmsi* (Csol), *Copidosoma floridanum* (Cflo), *Trichogramma brassicae* (Tbra), *Trichomalopsis sarcophagae* (Tsar), *Trichogramma pretiosum* (Tpre). Using TblastN (v2.2.26; E-value ≤ 1e−5), protein sequences were aligned to the *E. sophia* genome (Camacho et al., 2009), and GeneWise (v2.4.1) (Birney et al., 2004) software was employed to align matching proteins with homologous genomic sequences for accurate splice alignment and prediction of gene structures within each protein region. We constructed seven RNA-seq libraries, including different developmental stages of female *E. sophia* (600 eggs, *Bemisia tabaci* nymphs parasitized for <24 hours, dissected for host sampling; 200 first-instar larvae, *B. tabaci* nymphs parasitized for 48-60 hours, dissected for host sampling; 200 second-instar larvae, *B. tabaci* nymphs parasitized for 72-84 hours, dissected for host sampling; 80 third-instar larvae, *B. tabaci* nymphs parasitized for 120-132 hours, dissected for host sampling; 40 prepupae, *B. tabaci* nymphs parasitized for 168-178 hours, sampled after removing the host shell; 30 pupae, *B. tabaci* nymphs parasitized for 216-228 hours, sampled after removing the host shell; 50 adults, eclosed within <24 hours.). Total RNA extracted from the aforementioned samples were used for library preparation, and sequencing was performed on the Illumina NovaSeq 6000 platform (Cock et al., 2010). The sequencing output generated a total of 60.51G raw data, and after filtering, 59.88G clean data was used for genome annotation. Transcriptome assembly was performed using Trinity (v2.1.1) (Bolger et al., 2014) for genome annotation. To optimize genome annotation, RNA-Seq data from different tissues were extracted using Hisat (v2.0.4) (Kim et al., 2015) with default parameters to identify exonic regions and splice sites. The alignment results were used as input for Stringtie (v1.3.3)

(Pertea et al., 2015) with default parameters based on genome-guided transcriptome assembly. A non-redundant reference gene set was generated by merging genes predicted by the three methods using EvidenceModeler (EVM, v1.1.1) (Haas et al., 2008), incorporating masked transposable elements as inputs for gene prediction. A total of 14,914 protein-coding genes were predicted in *E. sophia* genome. The average length of predicted genes was 11,273.01 base pairs, with an average protein-coding region length of 1,451.53 bp. The average lengths of exons and introns were 275.58 and 2,301.66 bp, respectively. On average, each gene contained 5.27 exons (Table 4-10, Figure 4-2,3,4).

By using Blastp to align the protein sequences of *E. sophia* with Swiss-Prot (threshold E-value ≤ 1e−5), gene functions were assigned based on the best matches. InterProScan70 (v5.31) (Jones et al., 2008) was employed to annotate motifs and domains by searching public databases, including ProDom, PRINTS, Pfam, SMRT, PANTHER, and PROSITE. Gene Ontology (GO) IDs for each gene were assigned based on the corresponding InterPro entries. We mapped the genes to the NR20 database using the closest BLAST hits from the Swissprot20 database (Bairoch and Apweiler, 2000) (E-value <10-5) and DIAMOND (v0.8.22)/BLAST hits (E-value <10-5). Additionally, we mapped the genome to KEGG pathways (Kanehisa and Goto, 2000) and identified the best matches for each gene. Ultimately, 14,245 genes (95.5% of the total) in *E. sophia* genome were successfully annotated in at least one database (Table 4-11, Figure 4-5).

For the annotation of non-coding RNA (ncRNA) in the *E. sophia* genome, tRNAs were predicted using the tRNAscan-SE program (http://lowelab.ucsc.edu/tRNAscan-SE/) (Chan et al., 2021). As rRNAs are highly conserved, we opted for the rRNA sequences of related species as a reference and used Blast to predict rRNA sequences. Other ncRNAs, including miRNAs and snRNAs, were identified by searching the Rfam database (Kalvari et al., 2021) using the infernal software (http://infernal.janelia.org/) (Nawrocki and Eddy, 2013) with default parameters. In the end, a total of 1,457 non-coding RNAs were predicted, comprising 513 micro-RNAs (miRNAs), 514 transfer RNAs (tRNAs), 328 ribosomal RNAs (rRNAs), and 102 small nuclear RNAs (snRNAs) (Table 4-12).

Table 4-9. *Encarsia sophia* genome repeat sequence classification result statistics.

Repeat type	Denovo+Repbase		TE Proteins		Combined TEs	
	Length(bp)	% in Genome	Length(bp)	% in Genome	Length(bp)	% in Genome
DNA	26,727,384	6.71	5,680,941	1.43	28,604,198	7.18
LINE	12,742,058	3.20	5,263,490	1.32	15,788,154	3.96
SINE	73,932	0.02	0	0.00	73,932	0.02
LTR	135,720,939	34.08	19,125,339	4.80	137,771,019	34.59

Unknown	48,475,861	12.17	1,305	0.00	48,477,166	12.17
Total	213,763,901	53.67	30,069,622	7.55	214,739,217	53.92

Note: LINE (Long Interspersed Nuclear Elements): Long-dispersed repetitive sequences, with repeat unit lengths above 1000 bp; SINE (Short Interspersed Nuclear Elements): Short-dispersed repetitive sequences, with repeat unit lengths below 50 bp; LTR (Long Terminal Repeats): Sequences with long terminal repeats on both sides; Unknown: Indicates that the repeat sequence cannot be classified by RepeatMasker; Total: Non-redundant results obtained by removing overlapping portions between various classifications; Denovo+Repbase: Integrated results predicted by RepeatModeler, RepeatScout, Piler, and LTR_FINDER software, combined with the RepBase nucleic acid library, integrated using Uclust software according to the 80-80-80 principle, and annotated using RepeatMasker software to obtain transposon elements in the genome; TE proteins: Transposon elements obtained by annotating the genome separately with TE proteins based on the RepBase protein library using the RepeatProteinMask software; Combined TEs are the integrated results of the above two methods, after redundancy removal. This statistical result does not include the TRF identification results.

Table 4-10. *Encarsia sophia* statistical results of genome gene structure prediction.

	Gene set	**Number**	**Average transcript length(bp)**	**Average CDS length(bp)**	**Average exons per gene**	**Average exon length(bp)**	**Average intron length(bp)**
De novo	Augustus	15,956	8,411.35	1,489.68	4.99	298.43	1,733.98
	Glimmer HMM	33,861	10,499.07	761.97	3.49	218.60	3,917.21
	SNAP	23,924	23,075.70	887.93	7.08	125.46	3,650.71
	Geneid	31,571	5,185.88	928.60	3.29	282.56	1,861.97
	Genscan	21,484	11,826.00	1,330.44	5.30	250.93	2,439.65
Homolog	Cflo	10,815	6,875.42	1,389.21	4.89	284.35	1,411.96
	Tsar	11,077	5,268.28	1,326.65	4.62	287.03	1,088.25

	Tbra	7,750	5,957.41	1,246.99	4.23	295.03	1,459.88
	Nvit	11,634	7,063.07	1,451.86	4.99	290.80	1,405.37
	Csol	9,492	8,239.81	1,511.51	5.42	278.99	1,522.99
	Tpre	10,678	7,095.35	1,427.50	4.97	287.27	1,427.96
RNA seq	PASA	23,430	9,543.93	1,123.95	4.09	274.77	2,724.45
	Transcripts	51,252	14,530.86	2,300.38	4.39	524.37	3,611.08
EVM		17,419	8,973.75	1,359.08	4.86	279.83	1,974.37
Pasa-update*		17,270	10,398.83	1,374.41	4.88	281.68	2,326.25
Final set*		14,914	11,273.01	1,451.53	5.27	275.58	2,301.66

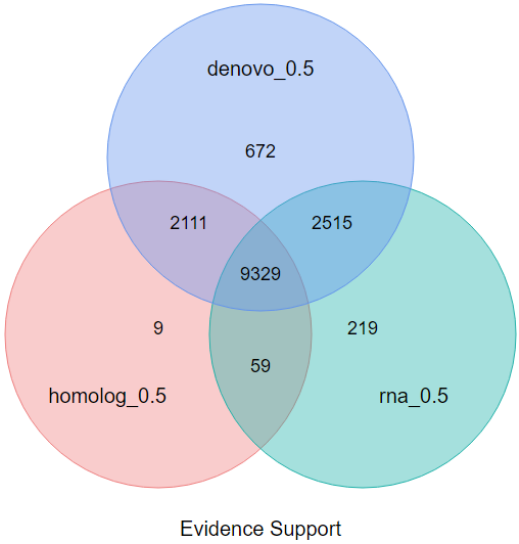


Figure 4-3. *Encarsia sophia* gene set evidence supports statistics.

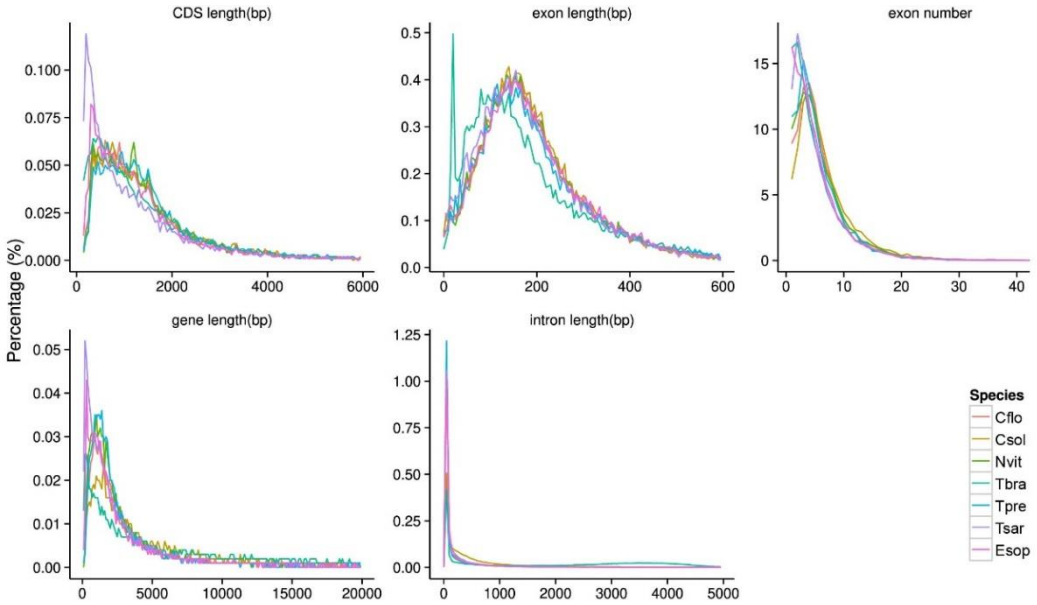


Figure 4-4. *Encarsia sophia* comparison diagram of various elements of genetically annotated closely related species.

Table 4-11. Functional annotation of *Encarsia sophia* proteins.

Type	Number	Percent(%)
Swissprot	10,110	67.80
Nr	13,514	90.60
KEGG	10,710	71.80
InterPro	13,363	89.60
GO	8,160	54.70
Pfam	10,103	67.70
Total annotated	14,245	95.50

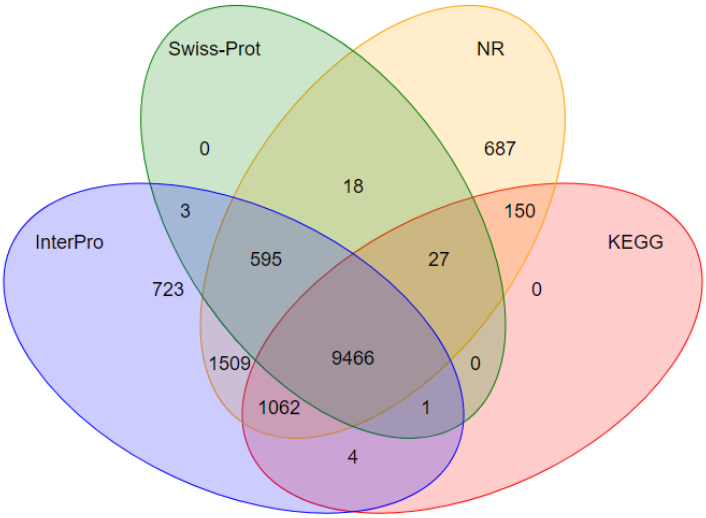


Figure 4-5. *Encarsia sophia* genome gene functional annotation statistical results

Table 4-12. *Encarsia sophia* genome non-coding RNA statistical results.

	Type	Copy number	Average length(bp)	Total length(bp)	% of genome
miRNA		513	146.54	75,174	0.018875
tRNA		514	74.33	38,206	0.009593
rRNA	rRNA	328	209.06	68,572	0.017217
	18S	95	289.37	27,490	0.006902
	28S	215	182.87	39,318	0.009872
	5.8S	18	98	1,764	0.000443
	5S	0	0	0	0
snRNA	snRNA	102	156.51	15,964	0.004008
	CD-box	15	146.40	2,196	0.000551
	HACA-box	11	188.09	2,069	0.000519
	splicing	75	154.25	11,569	0.002905
	scaRNA	1	130	130	0.000033
	Unknown	0	0	0	0

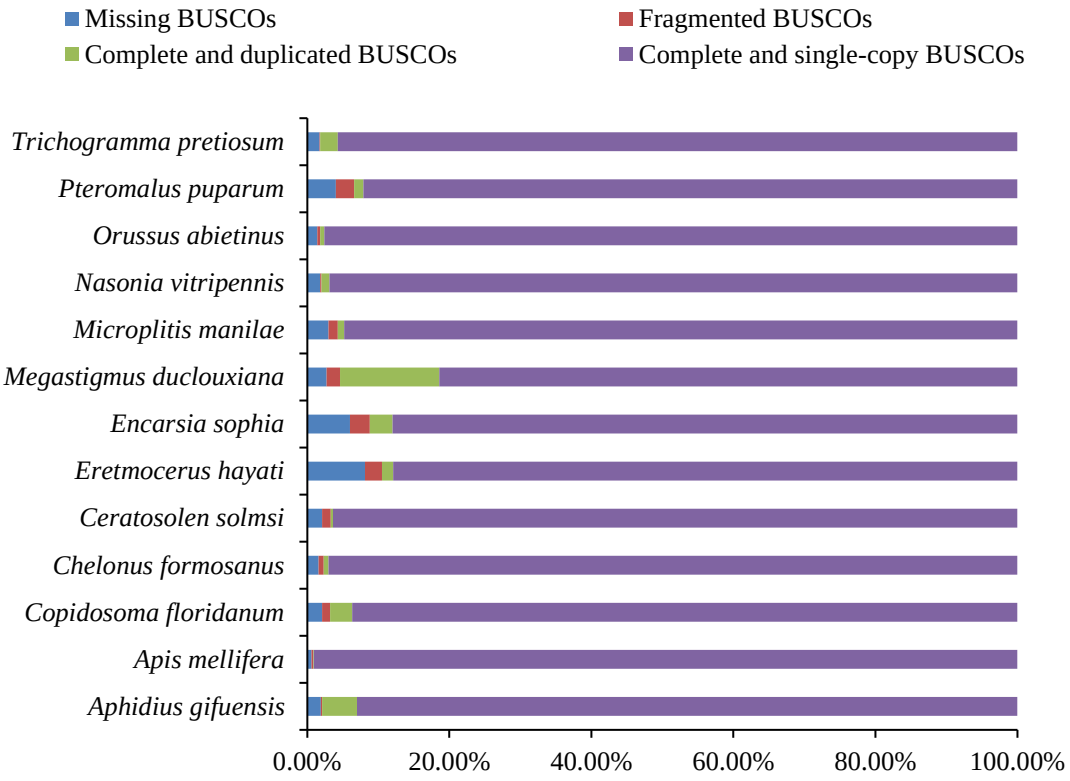
2.5 Phylogenetic analysis of gene families

We reconstructed phylogenetic trees for 13 species of Hymenoptera based on single-copy genes identified in the OrthoFinder (Emms and Kelly, 2019) results. Protein sequences from each gene family were independently aligned using MAFFT(v7) (Katoh and Standley, 2013), trimmed with default parameters using trimAl (v1.4)

(Capella-Gutiérrez et al., 2009), and then concatenated into a supersequence for phylogenetic analysis. The best model (VT + I + F) estimated by ProtTest3 (Nguyen et al., 2015) was used for maximum likelihood (ML) tree construction with bootstrap support using the RAxML package (Stamatakis, 2014). Divergence times were estimated using the MCMCTREE program in the PAML package (v4.9e) based on coding sequence (CDS). Three calibration time points were used based on fossil records (https://www.paleobiodb.org/), including *Orussus abietinus* (187.9–272.5 million years ago [Ma]), *Apis mellifera* (162.4–219.3 Ma), and *Aphidius gifuensis* (139.2–253.9 Ma). The resulting trees were visualized using FIGTREE (v1.4.4) (http://tree.bio.ed.ac.uk/software/figtree/).

We identified a total of 12,327 gene families, including single-copy, multi-copy, unique orthologs, other orthologs, and unclustered genes, in *E. sophia* and 12 other Hymenoptera species with high-quality genomes (Figure 4-6a) using OrthoFinder (Emms and Kelly, 2019) (Figure 4-6b). Utilizing 2560 single-copy genes, we revealed the phylogenetic relationships between *E. sophia* and the other 12 Hymenoptera species (Figure 4-6c). Phylogenetic analysis showed that *E. sophia* is most closely related to *Eretmocerus hayati*, with the divergence of *Copidosoma floridanum* from the Encyrtidae family occurring approximately 76.64 million years ago. *E. sophia*, *E. hayati*, *C. floridanum*, *Trichogramma pretiosum*, *Pteromalus puparum*, *Nasonia vitripennis*, *Ceratosolen solmsi*, and *Megastigmus duclouxiana* form a clade named Chalcidoidea, which is sister to Braconidae and diverged approximately 170.84 million years ago, consistent with previous phylogenetic studies (Peters et al., 2017; Li et al., 2021).

a


Missing BUSCOs
Fragmented BUSCOs
Complete and duplicated BUSCOs
Complete and single-copy BUSCOs
Trichogramma pretiosum
Pteromalus puparum
Orussus abietinus
Nasonia vitripennis
Microplitis manilae
Megastigmus duclouxiana
Encarsia sophia
Eretmocerus hayati
Ceratosolen solmsi
Chelonus formosanus
Copidosoma floridanum
Apis mellifera
Aphidius gifuensis
0.00%
20.00%
40.00%
60.00%
80.00%
100.00%

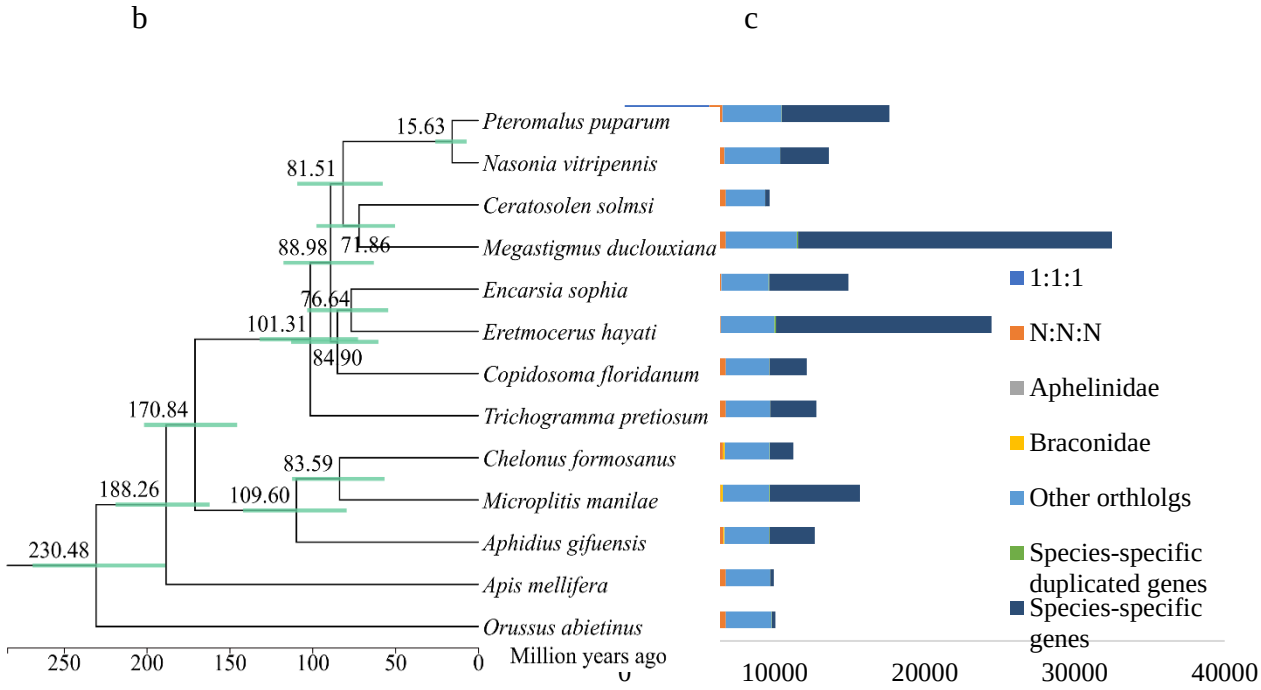


Figure 4-6. Phylogenetic tree of *Encarsia sophia* and 12 other Hymenoptera insects along with gene orthology statistics: **a,** BUSCO analysis of the 13 Hymenoptera species used for phylogenetic tree construction. **b**, Phylogenetic tree constructed using maximum likelihood method with species divergence times. **c**, Comparison of orthologous genes among the 13 Hymenoptera species, where 1:1:1 indicates common orthologs with the same copy in different species; N:N:N includes orthologous groups with different copy numbers in different species.

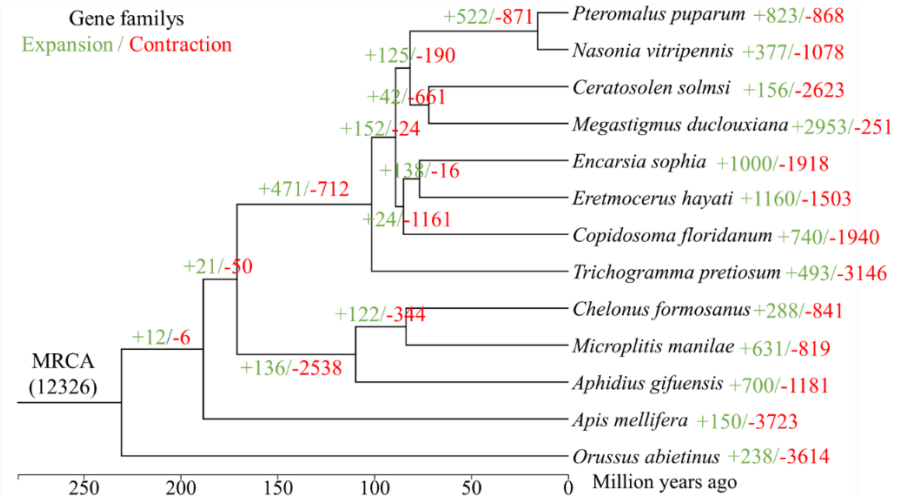
2.6 Expansion and contraction of gene families

We used the CAFÉ software (v4.2.1) (De Bie et al., 2006) to analyze the expansion and contraction of gene families. Gene family data from OrthoFinder (Emms and Kelly, 2019) and evolutionary trees with estimated divergence times between species were used as input. For gene families showing specific expansion or contraction, we conducted GO and KEGG pathway annotations using the Blast2GO (v5) and BlastKOALA (v2.2) online services. Enrichment analysis was performed using Omicshare cloudtools (http://www.omicshare.com/tools/?l=en-us). GO results were summarized and visualized using Revigo (http://revigo.irb.hr/) (Supek et al., 2011).

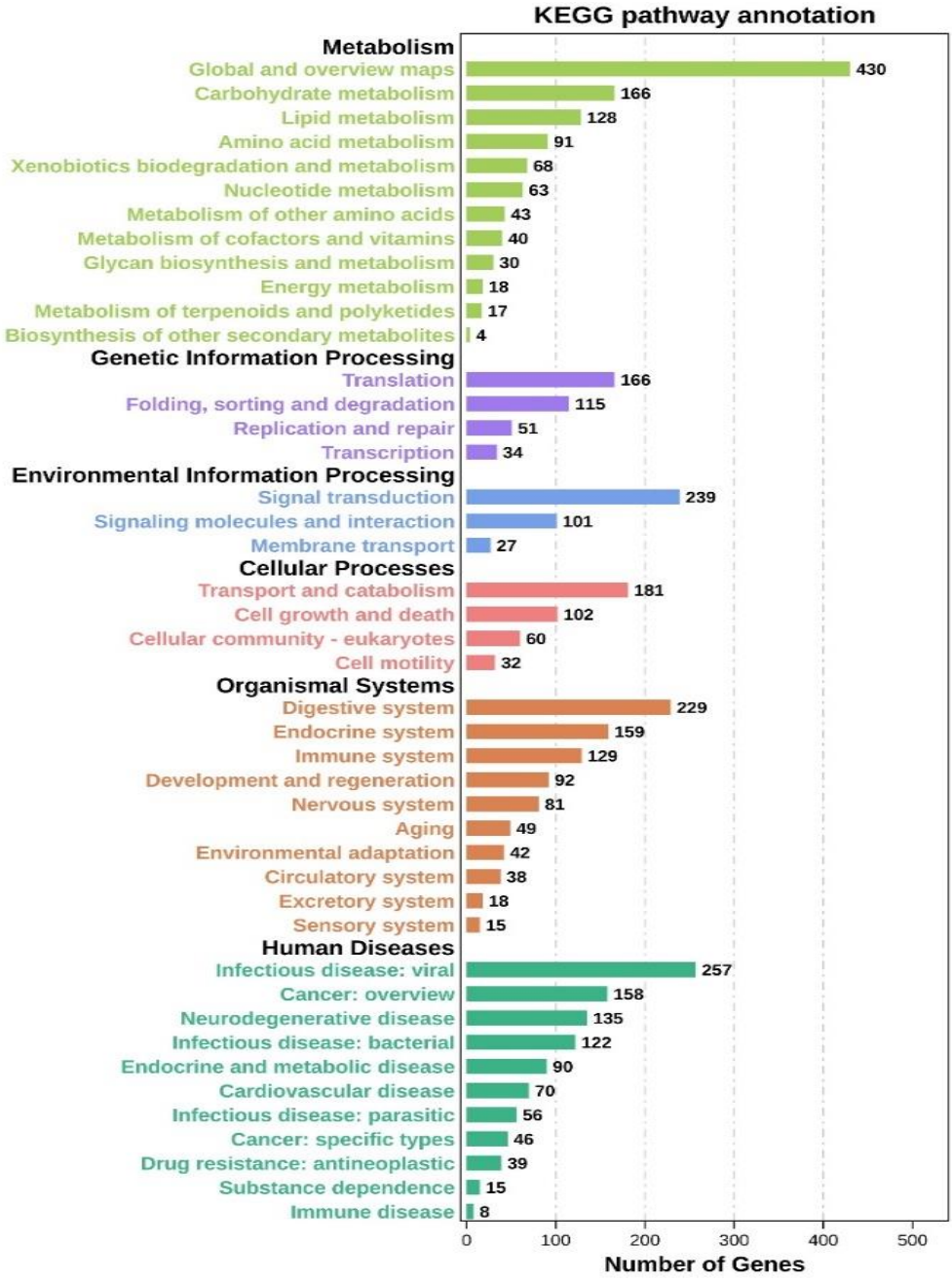
Using CAFÉ, we estimated gene family expansions and contractions in the *E. sophia* genome (Figure 4-7a) and conducted phylogenetic analysis with 12 other Hymenoptera insects. Significant expansions and contractions of gene families are often associated with species' adaptive evolution (Wu et al., 2019; Zhang et al., 2020). In the *E. sophia* genome, compared to the most recent common ancestor (MRCA) of

E. sophia and *E. hayati*, there were 1000 significantly expanded orthologous groups and 1918 significantly contracted orthologous groups (Viterbi $p < 0.05$). GO and KEGG enrichment analysis of expanded gene families showed enrichment primarily in proteolysis (GO:0006508, 255 genes, p = 1.2e-21), metabolic process (GO:0008152, 916 genes, p = 4.6e-08), oxidation-reduction process (GO:0055114, 197 genes, p = 1e-14), immune response (GO:0006955, 11 genes, p = 3.2e-05), protein metabolic process (GO:0019538, 393 genes, p = 6.3e-05), positive regulation of response to stimulus (GO:0048584, 17 genes, p = 8.9e-05), defense response (GO:0006952, 13 genes, p = 5.6e-4), sensory perception of chemical stimulus (GO:0007606, 37 genes, p = 3.365e-3), sensory perception (GO:0007600, 39 genes, p = 3.597e-3), metabolism of xenobiotics by cytochrome P450 (00980, 26 genes, p = 2.3e-5), AMPK signaling pathway (04152, 47 genes, p = 3.06e-4), fatty acid metabolism (01212, 41 genes, p = 3.29e-4), and ABC transporters (02010, 27 genes, p = 2.857e-3)(Figure 4-7b,c). Most of these are associated with immune defense pathways, metabolic pathways, and chemical perception systems.

a



b


KEGG pathway annotation
Metabolism
Global and overview maps 430
Carbohydrate metabolism 166
Lipid metabolism 128
Amino acid metabolism 91
Xenobiotics biodegradation and metabolism 68
Nucleotide metabolism 63
Metabolism of other amino acids 43
Metabolism of cofactors and vitamins 40
Glycan biosynthesis and metabolism 30
Energy metabolism 18
Metabolism of terpenoids and polyketides 17
Biosynthesis of other secondary metabolites 4
Genetic Information Processing
Translation 166
Folding, sorting and degradation 115
Replication and repair 51
Transcription 34
Environmental Information Processing
Signal transduction 239
Signaling molecules and interaction 101
Membrane transport 27
Cellular Processes
Transport and catabolism 181
Cell growth and death 102
Cellular community - eukaryotes 60
Cell motility 32
Organismal Systems
Digestive system 229
Endocrine system 159
Immune system 129
Development and regeneration 92
Nervous system 81
Aging 49
Environmental adaptation 42
Circulatory system 38
Excretory system 18
Sensory system 15
Human Diseases
Infectious disease: viral 257
Cancer: overview 158
Neurodegenerative disease 135
Infectious disease: bacterial 122
Endocrine and metabolic disease 90
Cardiovascular disease 70
Infectious disease: parasitic 56
Cancer: specific types 46
Drug resistance: antineoplastic 39
Substance dependence 15
Immune disease 8
0
100
200
300
400
500
Number of Genes

c Enriched GO Terms(Expansion)

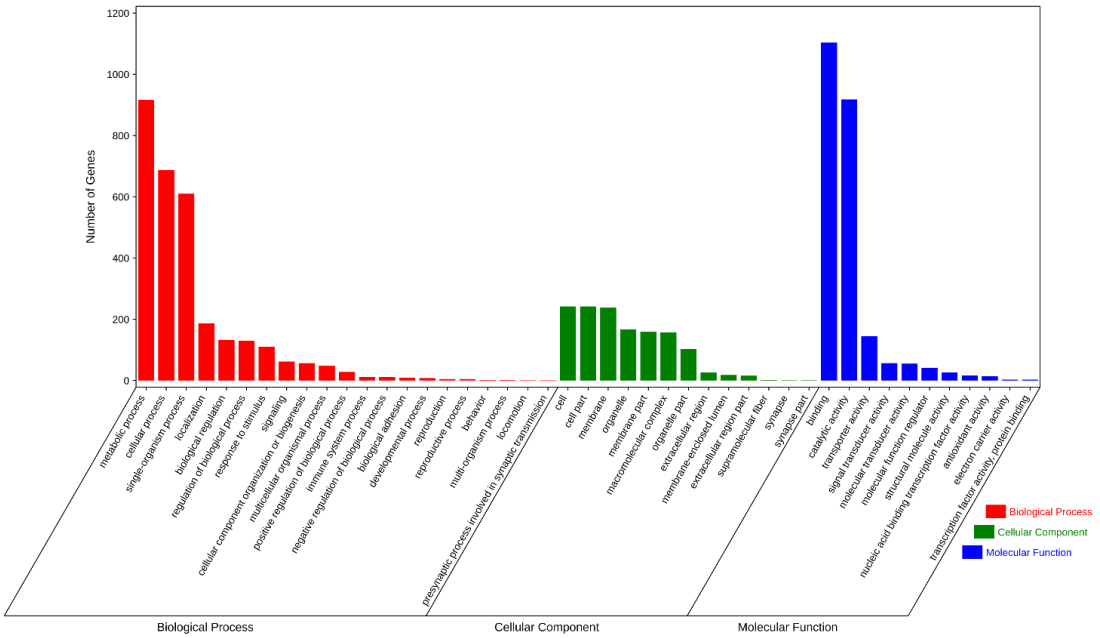


Figure 4-7. Evolution of gene families among *Encarsia sophia* and 12 other Hymenoptera species. **a**, The numbers behind each branch node on the phylogenetic tree represent the number of expanded (in green) and contracted (in red) gene families. MRCA, Most Recent Common Ancestor. **b**, KEGG enrichment analysis of significantly expanded gene families: The bar graph represents the number of genes involved in each KEGG pathway. **c**, GO enrichment analysis: BP, Biological process; CC, Cellular component; MF, Molecular function.

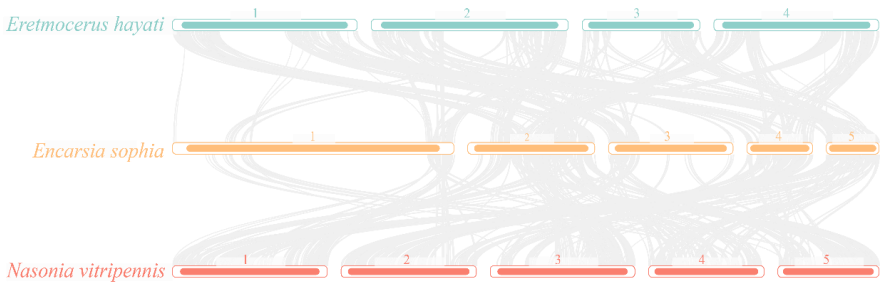
2.7 Chromosome synteny and identification of positively selected genes

To identify syntenic gene blocks among *E. sophia*, *E. hayati*, and *N. vitripennis*, we extracted coding sequences (CDS), searched for orthologous genes, and visualized high-quality gene blocks using the default parameters of MCscan (Multiple Collinearity Scan Toolkit) from JCVI (https://github.com/tanghaibao/jcvi).

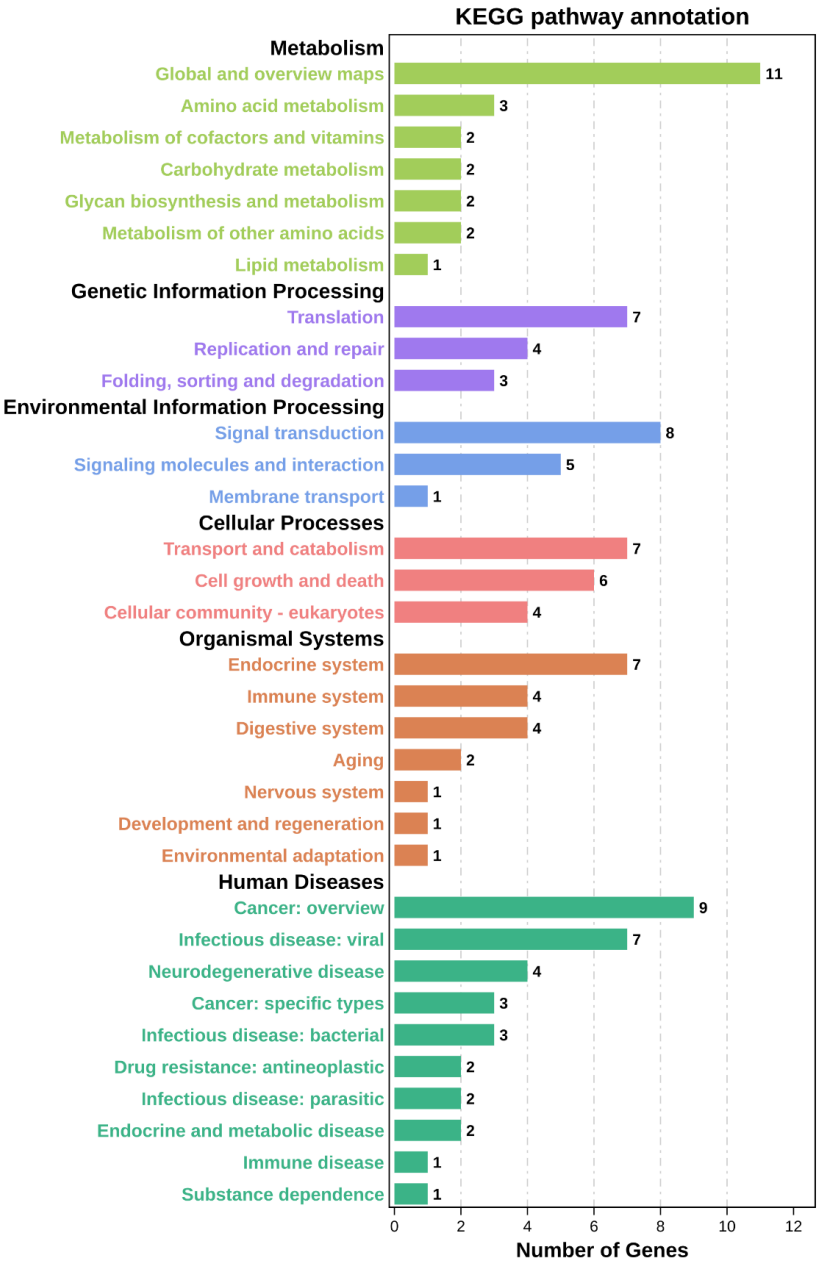
We defined at least 3 orthologous genes as a syntenic block. Between *E. sophia* and *E. hayati*, as a result, we identified 478 blocks, with the number of genes per block ranging from 4 to 58, averaging 12.02. Between *E. sophia* and *N. vitripennis*, we found 437 blocks, with the number of genes per block ranging from 4 to 76, averaging 13.29. The synteny relationships demonstrate conserved genome structures among these three species. Despite *E. sophia* being more closely related to *E. hayati*, the level of synteny between *E. sophia* and both *E. hayati* and *N. vitripennis* is similar (Figure 4-8a).

Using the branch model and branch-site model in codeml from PAML (v4.9e) (Yang, 2007), we analyzed positively selected genes (PSGs) in *E. sophia* by assessing the ratio of non-synonymous to synonymous substitutions (dN/dS, ω) for each single-copy gene. PSGs were identified at the single codon level by comparing null Model A (NSsites=2, model=2, fx_omega=1) with Model A (NSsites=2, model=2, fx_omega=0). The probabilities of amino acid positions with ω>1 were estimated using the Bayes Empirical Bayes (BEB) test in PAML (Zhang et al., 2012). Genes with positively selected sites and FDR-adjusted p-values less than 0.05 were determined as PSGs. We identified 105 positively selected genes in *E. sophia*. Through GO and KEGG enrichment analysis, significant terms include proline metabolic process (GO:0006560, 2 genes, p = 0.001), organic cyclic compound metabolic process (GO: 1901360, 19 genes, p = 0.010), heterocycle metabolic process (GO:0046483, 18 genes, p = 0.019), proteasomal protein catabolic process (GO:0010498, 2 genes, p = 0.025), cellular macromolecule catabolic process (GO:0044265, 4 genes, p = 0.0267), cellular aromatic compound metabolic process (GO:0006725, 17 genes, p = 0.037), Other glycan degradation (00511, 2 genes, p = 0.014), ECM-receptor interaction (04512, 3 genes, p = 0.027), Legionellosis (05134, 2 genes, p = 0.030) (Figure 4-8b,c). These genes are primarily associated with metabolic pathways, biosynthesis, and transportation, which may be crucial for utilizing different types of hosts in heteronomous hyperparasitism.

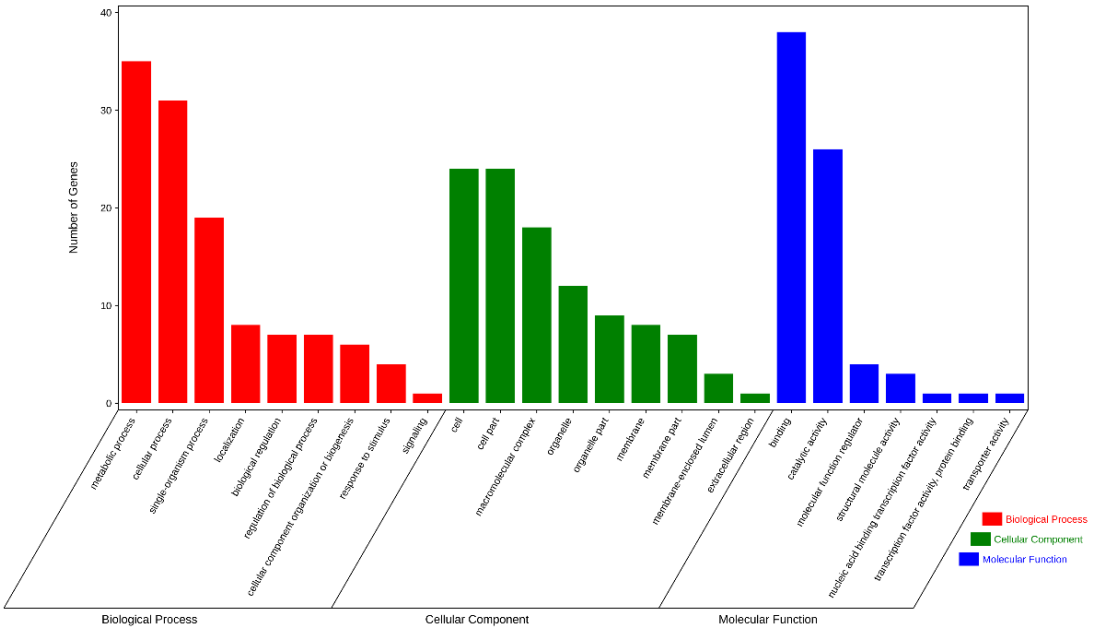
a



b


KEGG pathway annotation
Metabolism
Global and overview maps
11
Amino acid metabolism
3
Metabolism of cofactors and vitamins
2
Carbohydrate metabolism
2
Glycan biosynthesis and metabolism
2
Metabolism of other amino acids
2
Lipid metabolism
1
Genetic Information Processing
Translation
7
Replication and repair
4
Folding, sorting and degradation
3
Environmental Information Processing
Signal transduction
8
Signaling molecules and interaction
5
Membrane transport
1
Cellular Processes
Transport and catabolism
7
Cell growth and death
6
Cellular community - eukaryotes
4
Organismal Systems
Endocrine system
7
Immune system
4
Digestive system
4
Aging
2
Nervous system
1
Development and regeneration
1
Environmental adaptation
1
Human Diseases
Cancer: overview
9
Infectious disease: viral
7
Neurodegenerative disease
4
Cancer: specific types
3
Infectious disease: bacterial
3
Drug resistance: antineoplastic
2
Infectious disease: parasitic
2
Endocrine and metabolic disease
2
Immune disease
1
Substance dependence
1
0
2
4
6
8
10
12
Number of Genes

C


Number of Genes
0
10
20
30
40
metabolic process
cellular process
single-organism process
localization
biological regulation
regulation of biological process
cellular component organization or biogenesis
response to stimulus
signaling
cell
cell part
macromolecular complex
organelle
organelle part
membrane
membrane part
membrane-enclosed lumen
extracellular region
binding
catalytic activity
molecular function regulator
structural molecule activity
nucleic acid binding transcription factor activity
transcription factor activity, protein binding
transporter activity
Biological Process
Cellular Component
Molecular Function
Biological Process
Cellular Component
Molecular Function

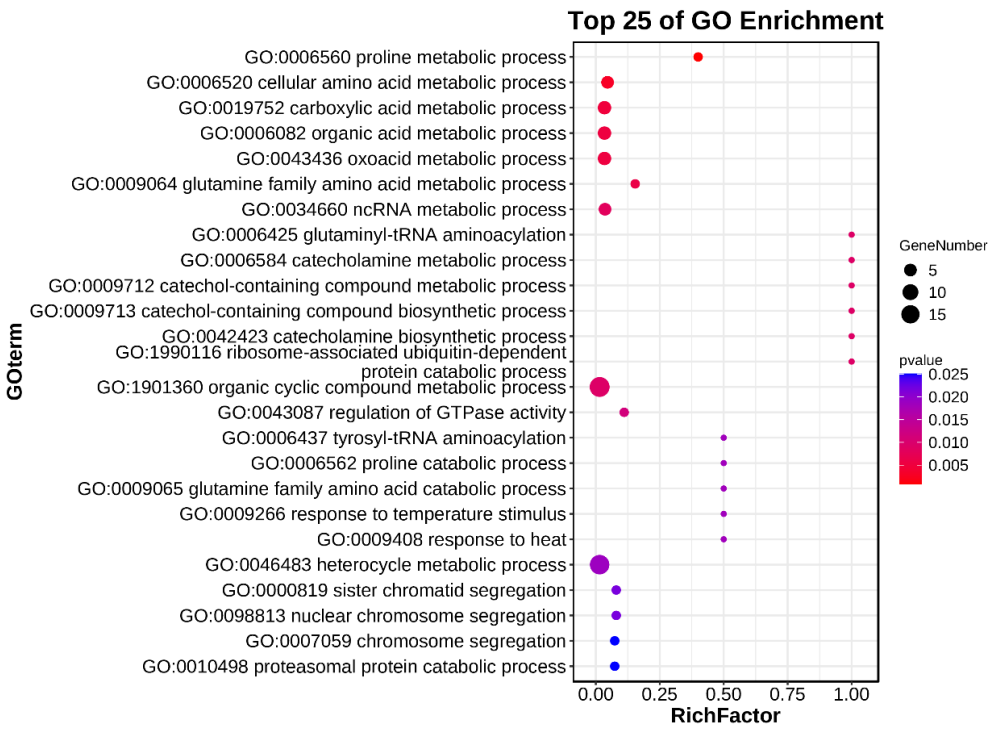
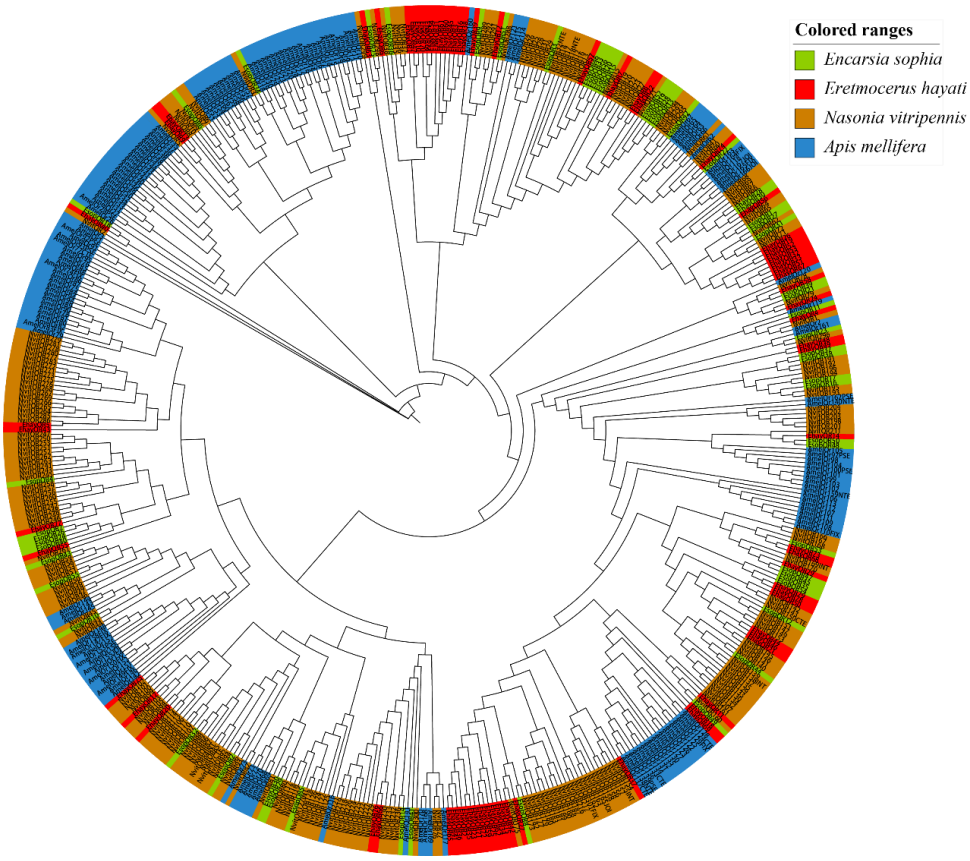

Top 25 of GO Enrichment
GOterm
GO:0006560 proline metabolic process
GO:0006520 cellular amino acid metabolic process
GO:0019752 carboxylic acid metabolic process
GO:0006082 organic acid metabolic process
GO:0043436 oxoacid metabolic process
GO:0009064 glutamine family amino acid metabolic process
GO:0034660 ncRNA metabolic process
GO:0006425 glutaminyl-tRNA aminoacylation
GO:0006584 catecholamine metabolic process
GO:0009712 catechol-containing compound metabolic process
GO:0009713 catechol-containing compound biosynthetic process
GO:0042423 catecholamine biosynthetic process
GO:1990116 ribosome-associated ubiquitin-dependent protein catabolic process
GO:1901360 organic cyclic compound metabolic process
GO:0043087 regulation of GTPase activity
GO:0006437 tyrosyl-tRNA aminoacylation
GO:0006562 proline catabolic process
GO:0009065 glutamine family amino acid catabolic process
GO:0009266 response to temperature stimulus
GO:0009408 response to heat
GO:0046483 heterocycle metabolic process
GO:0000819 sister chromatid segregation
GO:0098813 nuclear chromosome segregation
GO:0007059 chromosome segregation
GO:0010498 proteasomal protein catabolic process
0.00
0.25
0.50
0.75
1.00
RichFactor
GeneNumber
5
10
15
pvalue
0.025
0.020
0.015
0.010
0.005

Figure 4-8. Synteny and positive selection analysis of the *Encarsia sophia* genome. **a**, Synteny blocks between *Encarsia sophia*, *Eretmocerus hayati*, and *Nasonia vitripennis*. **b**, KEGG enrichment analysis of positively selected genes: The bar graph represents the number of genes involved in each KEGG pathway. **c**, GO enrichment analysis of positively selected genes: BP, Biological process; CC, Cellular component; MF, Molecular function. The size of the circles indicates the number of genes in the top 25 significantly enriched GO categories ($p < 0.05$) in BP.

2.8 Phylogenetic analysis of olfactory receptor genes.

The evolution of the heteronomous hyperparasitism between male and female *E. sophia* may be related to its olfactory recognition genes. To understand this unique and intriguing feature, we downloaded odorant receptors (ORs) protein sequences from UniProtKB and GeneBank to construct a reference database. Protein sequences predicted from the genomes of *E. sophia* and its closely related species *Eretmocerus hayati* were used as reference sequences for ORs in BLASTP searches. Then, Blast hits were retained for subsequent Pfam domain analysis using local hmmscan (Meng and Ji, 2013). Finally, genes with the odorant receptors conservative domains PF02949 or PF13853 were retained based on the hmmscan results (Yang et al., 2021). We used MAFFT(v7) (Katoh and Standley, 2013) for the alignment of ORs sequences. The alignment was trimmed through trimAl (v1.4) (Capella-Gutiérrez et al., 2009) with parameters set as "-automated1" (Misof et al., 2014). A maximum likelihood (ML) phylogenetic tree of odorant receptors genes from four species, *E. sophia*, *E. hayati*, *Nasonia vitripennis*, and *Apis mellifera*, was constructed using RAxML（v2）with parameters set as "-m PROTGAMMAJTTF " (Stamatakis, 2014). The model was estimated by ProtTest3 to be the best model (JTT + G + F), the bootstrap value was set to 1000 (Nguyen et al., 2015). The distribution of ORs genes on the *E. sophia* chromosome was visualized using TBtools-II (Chen et al., 2023). A total of 56 odorant receptors (ORs) genes were annotated in *E. sophia*. Possibly due to the smaller size of Aphelinidae species individuals, the number of ORs genes is fewer relative to other Hymenoptera species. Moreover, the homology is also lower, with sequence similarities ranging between15.72-73.04%. The distribution of ORs genes varies among different Hymenoptera species, forming multiple monophyletic branches, resulting in a highly diverse family of ORs genes. Some ORs in *E. sophia* and *E. hayati* clustered together on the phylogenetic tree, such as *EsopOR18*, *EsopOR22*, *EsopOR30*, *EsopOR39*(Figure 4-9a), indicating their potential relevance to the recognition of *B. tabaci*, as they are all parasitoids of *B. tabaci*. Additionally, specific ORs in *E. sophia* may be associated with the recognition of secondary hosts. Chromosome mapping results show that OR genes are distributed on all five chromosomes, with more genes on chromosomes 2, 3, 4, and 5, and fewer on chromosome 1, with only four genes distributed. There are five gene clusters containing three or more ORs genes on the five chromosomes (Figure 4-9b).

a


Colored ranges
Encarsia sophia
Eretmocerus hayati
Nasonia vitripennis
Apis mellifera

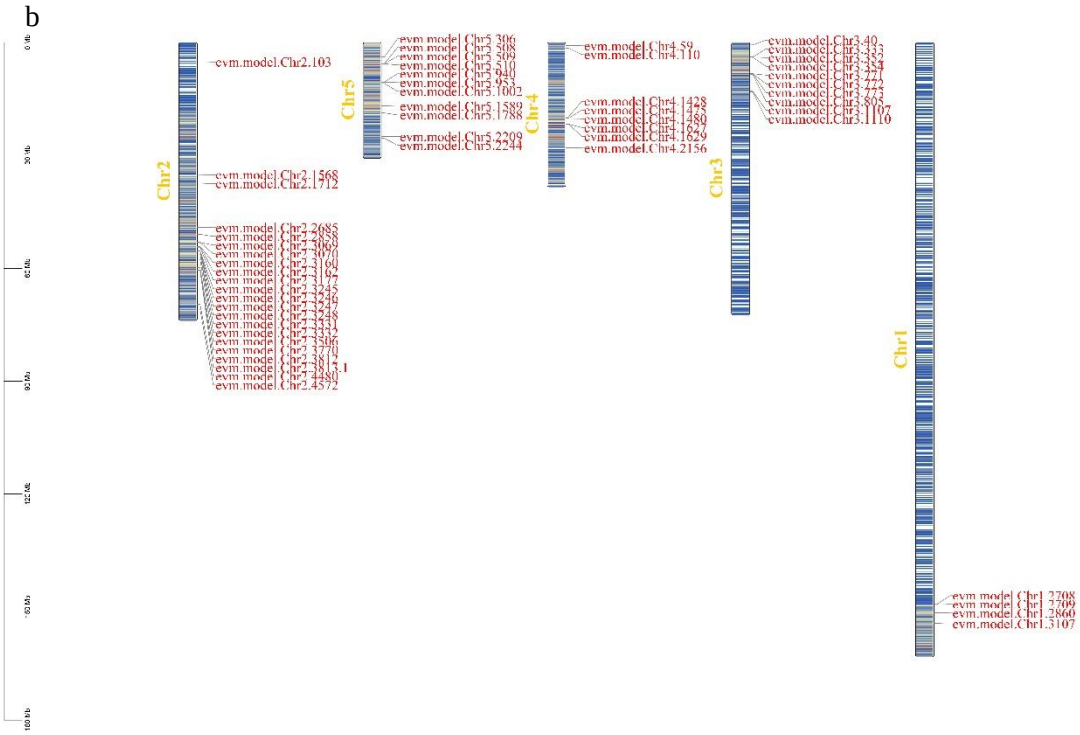


Figure 4-9. Phylogenetic analysis of odorant receptors (ORs) proteins from four Hymenoptera species and their genomic localization on chromosomes. **a.** Maximum likelihood phylogenetic tree of odorant receptors (ORs) genes from *Encarsia sophia*, *Eretmocerus hayati*, *Nasonia vitripennis*, and *Apis mellifera*. Species are grouped by different colors: *Encarsia sophia* (green), *Eretmocerus hayati* (red), *Nasonia vitripennis* (brown), and *Apis mellifera* (blue). **b.** Localization of ORs genes on chromosomes in *E. sophia*, with the density of chromosome genes displayed by stripes of different colors.

3. Data Records

The Illumina, PacBio, and Hi-C data for the *E. sophia* genome sequencing have been stored in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI, SRR29702816, SRR29702817, SRR29702818) and the Genome Sequence Archive (GSA) of the National Genomics Data Center (NGDC), under the accession numbers (NCBI: BioProject PRJNA1131600) and (NGDC: CRA017569), respectively. The transcriptome data used for annotation, covering various developmental stages of female *E. sophia*, have been stored in the SRA of NCBI and the GSA of NGDC: Egg (SRR29702811, CRR1218365), 1st instar larva (SRR29702815, CRR1218361), 2nd instar larva (SRR29702814, CRR1218362), 3rd instar larva (SRR29702813, CRR1218363), prepupa (SRR29702810, CRR1218366), pupa (SRR29702809, CRR1218367), and adult (SRR29702812,

CRR1218364). The access links are: [NCBI](https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1131600); [NGDC](https://bigd.big.ac.cn/gsa/browse/CRA017569). This Whole Genome Shotgun project has been deposited at GenBank under the accession JBFBOU000000000 https://www.ncbi.nlm.nih.gov/nuccore/JBFBOU000000000. The genome assembly and annotation files are available in figshare (https://doi.org/10.6084/m9.figshare.26426752).

4. Technical Validation

The quality, concentration, and integrity of DNA were measured using NanoDrop 2000&8000, Qubit 3.0 (Thermo Fisher Scientific, USA), and Agilent 4200 Bioanalyzer (Agilent Technologies, CA, USA), respectively. The integrity of RNA was assessed using the RNA Nano 6000 kit on the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). High-quality DNA and RNA were used for library preparation and sequencing. The sequence integrity of the assembled genome was evaluated using BUSCO (Benchmarking Universal Single-Copy Orthologs: http://busco.ezlab.org/) and CEGMA (Core Eukaryotic Genes Mapping Approach: http://korflab.ucdavis.edu/datasets/cegma/). Short fragment library reads were aligned to the assembled genome using BWA software (http://bio-bwa.sourceforge.net/), and the alignment rate, genome coverage, and depth distribution of reads were analyzed to assess the completeness of the assembly and the uniformity of sequencing. The genome’s Qv (quality value) was calculated using the Merqury-mash module (https://github.com/marbl/merqury) to evaluate the sequence accuracy of the assembled genome.

5. Code availability

Data processing was carried out according to the protocols and manuals of the relevant bioinformatics software, using default parameters unless otherwise specified. The versions and parameters of the software are described in the Methods section.

In **Chapter 3**, we investigated the ability of *Encarsia sophia* to regulate its offspring sex ratio under different host resource conditions. The results demonstrated that *E. sophia* optimizes its reproductive fitness by adjusting the sex ratio of its offspring, showing significant regulatory capabilities, particularly in response to changes in host density and the proportion of secondary hosts. This provides important behavioral evidence for understanding how heteronomous hyperparasitoids adapt their reproductive strategies in complex ecological environments. Subsequently, in **Chapter 4**, we conducted genome sequencing and high-quality assembly of *E. sophia*. Comparative analyses revealed divergence times between species, the expansion and contraction of gene families, and the identification of odorant receptor (OR) genes, offering deeper insights into the adaptive reproduction of *E. sophia*. **Chapter 5** then explored the specific mechanisms underlying these behaviors, with a focus on the molecular mechanisms driving oviposition decisions. Building on the genomic information from Chapter 4, we further analyzed how *E. sophia* uses olfactory cues to detect host volatiles and determine whether to lay female or male offspring. This progression, from behavioral regulation to molecular perception, allows for a more comprehensive understanding of *E. sophia*'s reproductive adaptability and its potential in biological control.

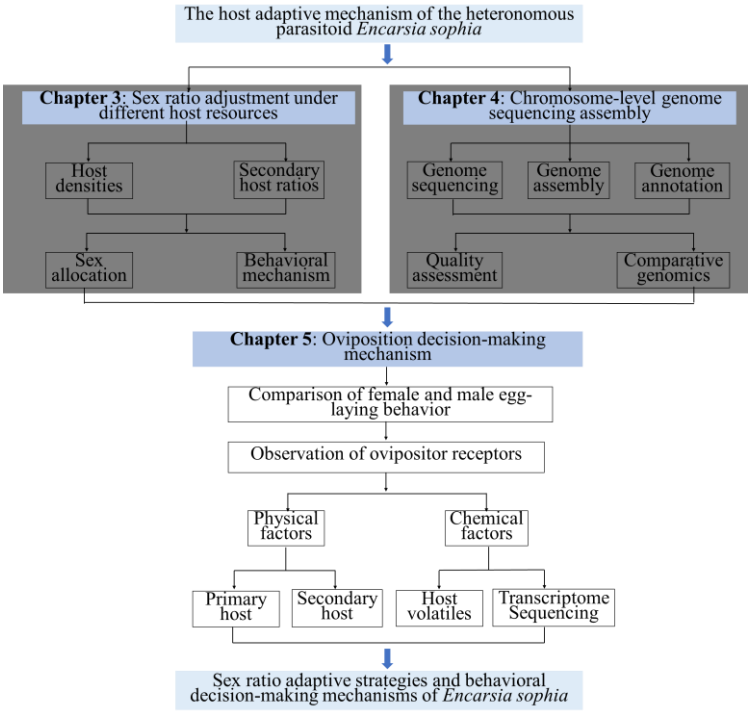


Figure 4-10. The transition from Chapter 3 and Chapter 4 to Chapter5 in the project "The host adaptive mechanism of the heteronomous parasitoid *Encarsia sophia*".

Chapter 5

Daughter or Son? Host Odor Determines Offspring Sex in Parasitoid

"*In biological control, we seek to work with nature, not against it, to manage the pests that threaten our crops, forests, and ecosystems.*"

Peter H. Raven

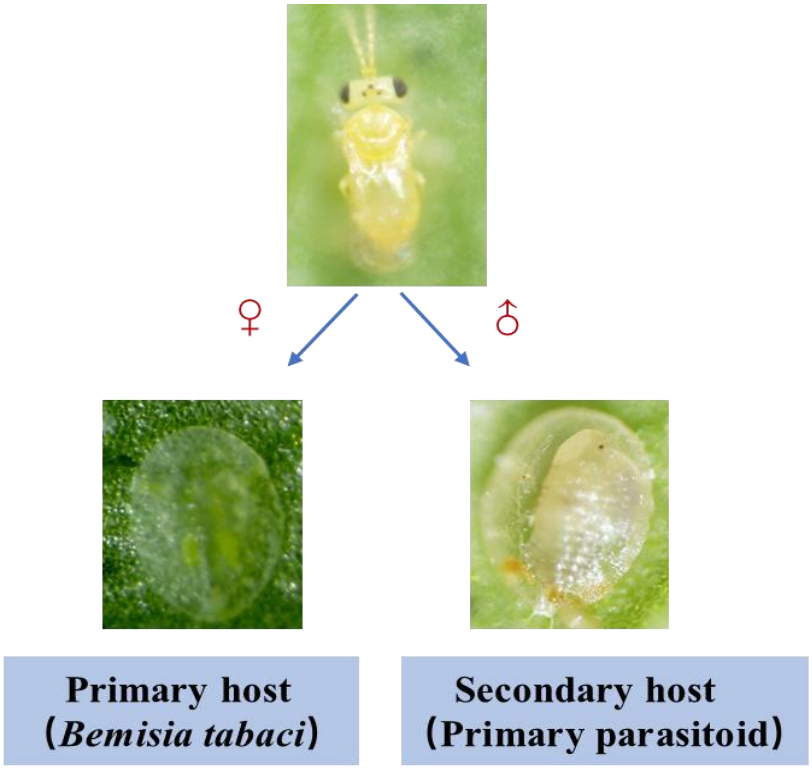


Figure 5-1. The host types for female and male production in *Encarsia sophia*.

Adapted from:

Man, X., Wu, S., Huang, C., Francis, F., Yang, N., Liu, W. Daughter or Son? Host Odor Determines Offspring Sex in Parasitoid. Submitted.

Graphical abstract

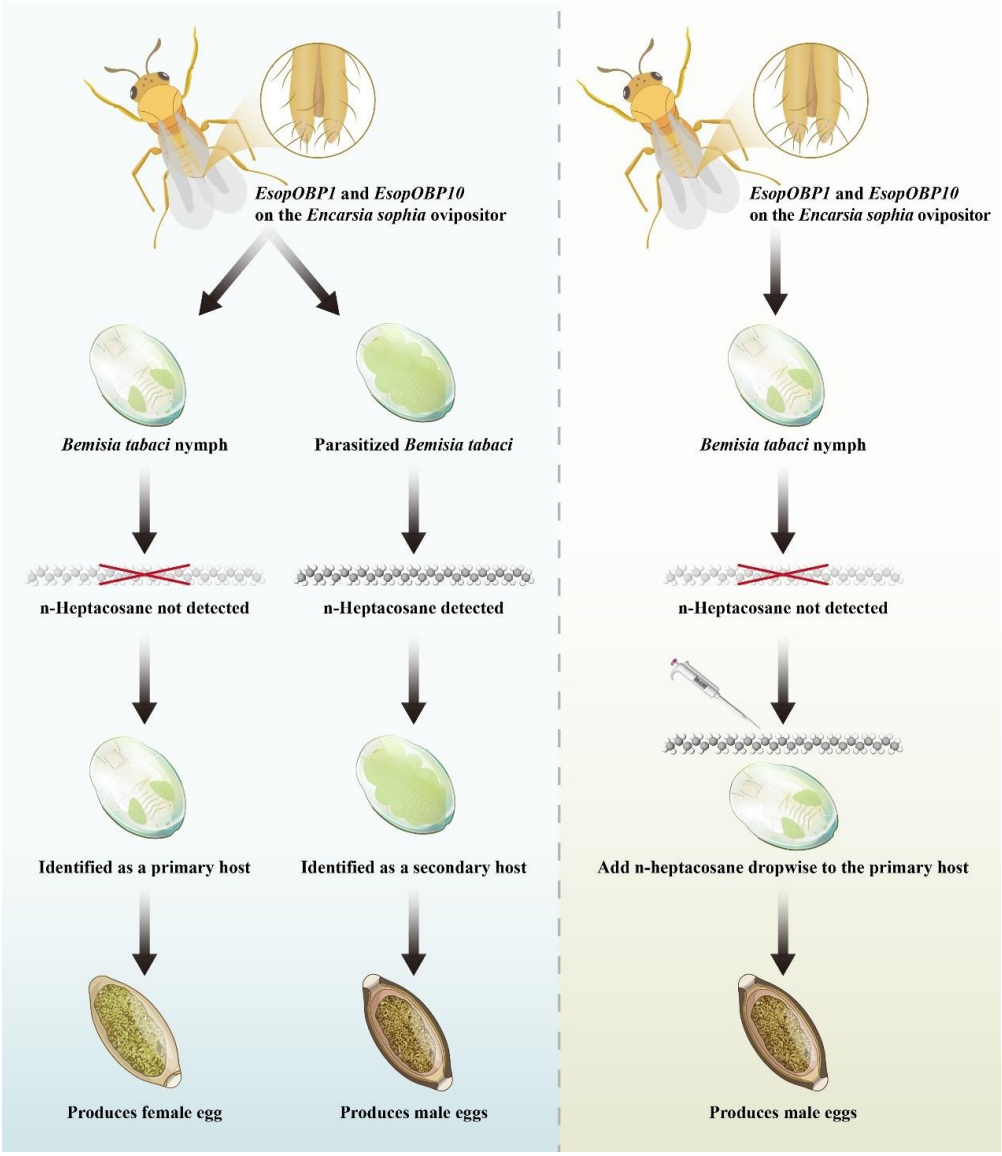


Figure 5-2. Graphical abstract of "Daughter or Son? Host Odor Determines Offspring Sex in Parasitoid".

Abstract

Heteronomous hyperparasitoids are unique biocontrol agents. Fertilized eggs are laid by mated females in primary hosts (target pests), developing into females, while unfertilized eggs are laid in secondary hosts (parasitoid larvae or pupae in previously parasitized pests), developing into males. How do females distinguish between primary and secondary hosts to lay sex-specific eggs? *Encarsia sophia*, a hyperparasitoid of the "super pest" *Bemisia tabaci*, uses *B. tabaci* for female progeny and various secondary hosts, including conspecific and heterospecific hosts parasitizing *B. tabaci* and aphids, for male progeny. This makes it an ideal model for studying the molecular mechanisms of heteronomous parasitism. In this study, the oviposition behavior of *E. sophia* females on primary and secondary hosts was observed and compared, and sensory receptors on the ovipositor were identified. First, physical factors were excluded by providing hosts with different mechanical pressures. Furthermore, previous views were overturned, revealing that the active movement of conspecific' larvae serves as self-protection against hyperparasitism rather than relying on the dryness of the host. Next, using hexane crude extracts and standard compounds in oviposition induction experiments, n-heptacosane was identified as the key compound for male production in secondary hosts. Subsequently, transcriptome sequencing, gene expression studies, and whole-mount in situ hybridization revealed that *EsopOBP1* and *EsopOBP10* are highly expressed in the ovipositor. By testing the binding ability of these genes with differential compounds and conducting behavioral assays with mixtures of binding-capable compounds, the crucial olfactory role of the ovipositor in host oviposition decision-making was ultimately demonstrated. This study is the first to elucidate the mechanism behind oviposition decision in heteronomous hyperparasitoid and the first to identify functional genes on the ovipositor of Hymenoptera specie, demonstrating the critical role of the ovipositor in host recognition. By treating hosts with oviposition compounds, supporting the transformation of hyperparasitic behavior in male production, reducing interspecific competition, and enabling unmated females to contribute to biocontrol efforts.

***Keywords*:** heteronomous hyperparasitoid, Oviposition decision, primary host, secondary host, Volatiles, odorant-binding proteins

1. Introduction

In the field of insect sex determination, it is widely recognized that two principal mechanisms exist: one governed by environmental cues and the other by genetic factors. For Hymenoptera insects, sex determination is typically controlled by genetics, specifically through a haplodiploid system. However, a particularly intriguing subset within this group—the heteronomous hyperparasitoids—exhibits a unique adaptation wherein the development of male and female offspring is linked to different host types. Our findings reveal that female wasps in this subset can determine the sex of their offspring by detecting the odor of the host. In essence, this suggests that the host's odor, an environmental factor, may function as a critical "switch" that determines whether the eggs are fertilized.

Hymenoptera parasitoids' ability to successfully utilize cues that indicate the location of host habitats and to distinguish between suitable and unsuitable hosts is crucial for their efficiency in the wild (Wajnberg et al., 2008; 2013; Bichang'a et al., 2018). During the search for suitable hosts, parasitoids typically rely on long-range and short-range stimuli emitted from the host habitat (Vinson, 1975,1976; Godfray, 1994), followed by stimuli directly associated with the host and its products (Vinson, 1985; Vet and Dicke, 1992). Most parasitoids only need to distinguish between hosts and non-hosts. However, there is a special category of parasitoids, the heteronomous hyperparasitoids, where male and female individuals have different host relationships. Females are primary parasitoids, mainly parasitizing Hemiptera insects such as aphids, whiteflies, and scale insects (primary hosts), while males are hyperparasitoids that develop by using the larvae of conspecific or heterospecific parasitoids within the primary host (secondary hosts) (Walter, 1983; Williams, 1996; Hunter and Woolley, 2001). These parasitoids not only need to distinguish between hosts and non-hosts but, more importantly, they must also differentiate between primary hosts for female production and secondary hosts for male production. Although the application of such parasitoids is increasing (Shahbazvar et al., 2022; Tize et al., 2023; Zhang et al., 2023), current research on these parasitoids remains at the level of basic biological and behavioral studies (Xu et al., 2018; Kidane et al., 2020; Zhao et al., 2022). It is still unclear how they distinguish between primary and secondary hosts. This distinguishing ability is a key characteristic that differentiates these parasitoids from other types, and understanding this feature not only helps in understanding their evolution but also makes it possible to regulate secondary hosts for male production and reduce interspecific competition.

The host selection process and final acceptance behavior of parasitoids begin with the analysis of external cues and culminate with probing and subsequent oviposition. This complex behavioral sequence to assess host suitability is regulated by various physical and chemical factors (Larocca et al., 2007). Typically, physical cues such as host size (Shirota et al., 1983; Kouamé and Mackauer, 1991), host cuticle texture (Arthur, 1981), shape (Vinson, 1985), and color (Ankersmit et al., 1981,1986; Michaud and Mackauer, 1994,1995; Battaglia et al., 2000) influence the oviposition behavior of females. Chemical cues, such as herbivore-induced plant volatiles

(HIPVs), green leaf volatiles (GLVs), and host-released pheromones (Battaglia et al., 1993; De et al., 1998; Buitenhuis et al., 2004; Dong et al., 2008; Jiang et al., 2022), also play a significant role in female oviposition behavior. Parasitoids determine their hosts primarily through antennal drumming and ovipositor probing. As essential sensory organs, the antennae and ovipositors are equipped with various types of sensilla that play a crucial role in detecting external information during host searching and oviposition recognition processes (Weseloh, 1972; Dahms, 1984; Bin and Vinson et al., 1986; Pang et al., 2020; Zhu et al., 2021). Depending on their physiological functions, insect sensilla can be categorized into chemosensilla, mechanosensilla, thermosensilla, and hygroreceptors (Slifer, 1970; Steinbrecht, 1997; Keil, 1999). *Trichogramma chilonis* can use their antennae to sense pheromones deposited on the host surface to decide whether to oviposit (Wang et al., 2016). *Diachasmimorpha longicaudata* females use their antennae to detect volatile organic compounds (VOCs) released by fruit flies and their hosts for oviposition (Wulff et al., 2024). The ovipositor of parasitoids is equipped with numerous sensory organs derived from glandular tissues (Snodgrass, 1931,1935), playing a crucial role in locating, recognizing, and accepting suitable hosts, as well as in the oviposition process (Papp, 1974; Le et al., 1996). *Leptopilina heterotoma* has gustatory structures at the tip of the female ovipositor, which generate different electrophysiological signals upon contact with the hemolymph of parasitized and non-parasitized *Drosophila*, enabling the parasitoid to detect parasitized hosts (van et al., 2007). The aphid parasitoid *Aphidius ervi* possesses multiporous chemosensilla on its ovipositor that can detect chemical signals in the host hemolymph. Females rarely oviposit in aphids filled with host hemolymph unless these aphids are coated with cornicle secretion (Larocca et al., 2007). There have been many studies on chemosensory genes on insect antennae, but the identification of chemosensory genes on ovipositors has only been reported in a few cases, such as in the Diptera *Bactrocera dorsalis* (Hendel) and the Lepidoptera *Helicoverpa assulta*. However, in Hymenoptera ovipositors, these genes have not yet been characterized (Li et al., 2020; He et al., 2022; Xu et al., 2024).

We used the typical heteronomous hyperparasitoid *E. sophia*, a dominant parasitoid of the "super pest" *B. tabaci*, to observe the heteronomous oviposition behavior. This clarified the role of the female's ovipositor in oviposition decisions on the corresponding host. Electron microscopy was then employed to observe the physical and chemical olfactory sensilla on the ovipositor. The influence of physical and chemical factors of the host on the oviposition decisions of *E. sophia* was separately investigated. Firstly, the physical differences (mechanical pressure) between primary and secondary hosts were used to exclude the impact of this factor on the oviposition decisions of females. Interestingly, it was discovered that the active movement of conspecific species' larvae provides self-protection against hyperparasitism rather than the host's dryness status proposed by Hunter and Kelly. Observations of dead secondary hosts, which cannot produce male eggs, and primary hosts, which are used for female egg production, demonstrated that chemical factors influence oviposition decisions. This was evidenced by the production of male eggs in both types of hosts treated with n-hexane extracts from secondary hosts. Further, leveraging the

characteristic that *E. sophia* can produce male eggs in various secondary hosts but only female eggs in one primary host, and combining this with behavioral experiments using compound standards, the key male-inducing compound in secondary hosts, n-heptacosane, was identified. Transcriptome sequencing analysis, qPCR, and whole-mount in situ hybridization experiments of female wasp antennae and ovipositor pinpointed the key odorant-binding protein genes, *EsopOBP1* and *EsopOBP10*, on the ovipositor. Fluorescence competitive binding and molecular docking confirmed the binding interaction between these genes and the key compounds. Finally, the induction of male egg production by mixtures of binding compounds was tested, once again proving the inducible effect of n-heptacosane on females' behavior. This study ultimately revealed the significant olfactory role and mechanism of the *E. sophia* ovipositor in host oviposition decisions.

2. Experimental model and study participant details

2.1 Insect culture and host plant

E. sophia was introduced in 2008 to the Biological Invasion Laboratory of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, from the Vegetable Pest Integrated Management Laboratory at the University of Texas, USA. It is reared in the air-conditioned insectary of the Langfang Research and Testing Base of the Chinese Academy of Agricultural Sciences, with *B. tabaci* MEAM1 as the host. The *B. tabaci* MEAM1 population originated from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, with no history of pesticide use. The host plant is cotton (*Gossypium* spp.), and the variety is Zhongmian 49. All insect experimental materials were reared under conditions of 26 ± 1°C, 65 ± 5% RH, and a 14L:10D photoperiod in a climate chamber (Ningbo Safe, China).

2.2 Comparison of the behavior of *Encarsia sophia* in producing females in primary hosts and males in secondary hosts

To provide mated and unmated females with primary hosts (3rd-4th instar nymphs of *B. tabaci*) and secondary hosts (*E. sophia* larvae at the third instar to pre-pupal stage), observe the handling behavior of females towards parasitizing hosts, and record the duration of each behavior process. Observations were repeated for 20 sets, respectively.

2.3 Observation of the ovipositor sensory types in *Encarsia sophia*

We photographed and observed the ovipositor of *E. sophia* using scanning electron microscopy. Due to the small size of the parasitoid wasp, to prevent sample loss during processing, we adopted a strategy of first treating the whole sample and then dissecting and photographing specific parts. The specific steps were as follows:

Sampling: live female adults were placed in a 1.5 mL centrifuge tube, sealed with absorbent cotton, and frozen in the refrigerator for 10 minutes. Cleaning: samples

were cleaned for 2 minutes in a CNC ultrasonic cleaner with distilled water at a frequency of 50 Hz. The samples were observed under a stereomicroscope to check for surface contaminants. If present, the cleaning process was continued until no contaminants were visible. The total cleaning time was kept within 10 minutes to prevent sample damage. After cleaning, the samples were placed in 2.5% glutaraldehyde-phosphate buffer solution (pH = 7.2) and fixed at 4°C for 24 hours. Dehydration: samples were dehydrated by sequential immersion in alcohol with concentrations of 30%, 50%, 70%, 80%, 90%, 95%, and 100%, each repeated three times for 15 minutes. Drying: critical point drying with CO_2 for 1 hour. Mounting and coating: after drying, the samples were carefully removed from the specimen, and the ovipositor of the female was oriented facing up and fixed on the sample stage using double-sided tape. Gold coating was performed using a Leica EM ACE600 ion sputtering coater. Scanning electron microscope observation: observations were conducted using a Regulus 8100 high-resolution field emission scanning electron microscope at an accelerating voltage of 10 kV, and photographs were taken.

2.4 Physical factors in the oviposition decision-making of female and male offspring in *Encarsia sophia*

Selecting young (2nd instar) and mature (4th instar) nymphs of *B. tabaci*, representing different hemolymph states (the hemolymph volume increases gradually from the 2nd to the 4th instar, representing a process of increasing oviposition mechanical pressure), mated females *E. sophia* were separately provided, released for 2 hours, and the oviposition rate on the host was recorded on 20 replicates.

Selecting different developmental stages of *Eretmocerus hayati* (larval stage - prepupal stage - pupal stage, with decreasing hemolymph in the host, representing a process of decreasing oviposition mechanical pressure), virgin females *E. sophia* was provided separately. After releasing the wasps for 2 hours, the oviposition rate on the host was recorded over 20 times repeatedly.

To investigate the reason why *E. sophia* in its 3rd instar larval stage cannot be parasitized, we dissected and compared two secondary hosts that had developed to the same larval stage (*E. sophia* 3rd instar larvae and *E. hayati* 3rd instar larvae). After dissection, it was clearly observed that *E. sophia* larvae were more active and constantly moving (Figure 2, D), while *E. hayati* larvae were almost immobile (Figure 2, E). Based on this observation, we designed a cold treatment experiment for the host of *E. sophia* 3rd instar larvae to reduce larval movement and then observed whether the host could be parasitized: cotton leaf discs with *E. sophia* 3rd instar larvae were placed at 4°C for 2 hours, while leaf discs with secondary hosts of the same age from the same leaf were kept at 26°C as a control. Five unmated *E. sophia* females were introduced to both the cold-treated and non-treated leaf discs simultaneously. After 2 hours of oviposition, the hosts were dissected to calculate the oviposition rate. The experiment was repeated with 20 replicates.

2.5 Identification and screening of compounds in the host associated with oviposition

2.5.1 Collection of host volatiles

Considering that the host volatiles influencing the oviposition decision of the female wasp should belong to contact volatiles, we employed n-hexane extraction to collect host volatiles.

Primary host volatiles

Using the n-hexane solvent extraction method, *B. tabaci* nymphs were extracted at different time intervals (1 min, 5 min, 30 min, 60 min): 400 3rd - 4th instar *B. tabaci* nymphs were picked with a dissecting needle and placed into a 1.5 mL vial. Subsequently, 100 μL of n-hexane (chromatographically pure) solution was added, and the mixture was shaken thoroughly. After soaking in the dark at room temperature, the extract was filtered through a 0.22 μm filter membrane into a new vial and stored in a refrigerator at 4°C for later use.

Secondary host volatiles

Using the n-hexane solvent extraction method, different time gradients (1 min, 5 min, 30 min, 60 min) of extraction were performed on *E. sophia* prepupae, *Encarsia formosa* prepupae, *E. hayati* prepupae and *Aphidius gifuensis* larvae: 400 *E. sophia* prepupae, *E. formosa* prepupae, and *E. hayati* prepupae were picked with a dissecting needle into separate 1.5 mL vials, and then 100 μL of n-hexane (chromatographically pure) solution was added to each. For 200 *A. gifuensis* aphid mummies, they were gently brushed into a 1.5 mL vial, followed by the addition of 200 μL of n-hexane (chromatographically pure) solution. After thorough shaking and soaking in the dark at room temperature, the extracts were filtered through a 0.22 μm filter membrane into new vials and stored in a refrigerator at 4°C for later use.

2.5.2 Oviposition behavior in female *Encarsia sophia* to secondary host extracts

The 3rd to 4th instar nymphs of *B. tabaci* were soaked in a crude extract from the secondary host and proposed to *E. sophia* virgin female was for 2 hours. The oviposition rate of the treated hosts was recorded. As controls, untreated and n-hexane-treated healthy nymphs of the same age were used. A total of 30 replicates were set up (with each petri dish as a unit, containing 7-10 hosts per dish).

2.5.3 Identification and analysis of specific volatile compounds from secondary hosts

Based on the results of the induction of male egg production in female wasps by the crude extract, the 5-minute extracts of both primary and secondary hosts were analyzed using GC-MS (Shimadzu, Tokyo, Japan) with a DB-5MS column (30 m, 0.25 mm, 0.25 μm). A 1 μL aliquot of the extract was injected, with helium gas at a flow rate of 1.2 mL/min used as the carrier. The initial temperature was set at 35 ℃ for 5 min, then increased at a rate of 10 ℃/min to 280 ℃ and held for 20.5 min. The ion source temperature was 250 ℃, and the interface temperature was 270 ℃. Compounds were preliminarily identified based on their retention times, and comparison with the NIST17 mass spectral library and retention times of straight-

chain alkane standards (C8-C33) (Ayelo et al., 2022). Six replicates of each extract under each treatment were analyzed. Screening for compounds absent in primary hosts and common to different types of secondary hosts.

2.5.4 Induction male egg production in *Encarsia sophia* by secondary hosts

The standardized compounds, which were absent in the selected primary host but common among different types of secondary hosts, were dissolved in hexane to prepare standard solutions at concentrations of 0.1, 1, 10, and 100 μg/mL. These standard solutions were then applied to 3rd to 4th instar nymphs of *B. tabaci*. Subsequently, virgin female *E. sophia* was given 2 hours to interact with the hosts, after which the hosts were dissected to calculate the oviposition rate. This process was repeated 30 times (as in the previous method, using petri dishes as units), with hexane used as a control.

2.6 Sample collection and transcriptome sequencing

Under a stereomicroscope, the newly emerged *E. sophia* female within 24 hours were placed on glass slides containing 1× PBS buffer (pH 7.2-7.4) for dissection of antennae (including the head) and ovipositors using dissecting needles. There were 400 pairs of antennae per sample and 4000 individuals for ovipositors per sample, with three biological replicates. Dissection needles and glass slides were soaked in 75% alcohol before use, and dissected tissues were immediately placed in liquid nitrogen and stored at -80°C.

According to the protocol, total RNA from all collected samples was extracted using Trizol reagent (Invitrogen, USA). RNA integrity and contamination were assessed using 1% agarose gel electrophoresis. The purity of RNA was checked using a Nanodrop spectrophotometer (IMLEN, CA, USA). RNA concentration was measured using the Qubit RNA Analysis Kit and Qubit 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was evaluated using the RNA Nano6000 Assay Kit with the Agilent 2100 system. Illumina sequencing of the samples was conducted by Novogene (China). The cDNA libraries were prepared using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina according to the manufacturer's instructions (NEB, USA). To ensure the quality and reliability of data analysis, raw data were filtered to remove reads with adapters, reads containing N bases (representing undetermined nucleotides), and low-quality reads (reads with more than 50% of bases with Qphred <= 20). Additionally, the Q20, Q30, and GC content were calculated for clean data. All subsequent analyses were conducted based on high-quality clean data. The transcriptome was assembled using the Trinity software package, and high-quality clean reads were aligned to the reference gene sequences using HISAT2 v2.0.5. The expression levels of genes were calculated and normalized using the Fragments Per Kilobase Million (FPKM) method, and differential expression analysis of genes was performed using the DEGSeq2 R package (1.20.0). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were used to assign DEGs to functional categories based on respective databases, with adjustment of the resulting P-values using the Benjamini and Hochberg method to control the

false discovery rate. Genes with an adjusted P-value <= 0.05 as determined by DESeq2 were considered differentially expressed.

2.7 Cloning and sequence analysis of OBPs genes

Based on the above results, we selected the OBPs genes with significantly higher expression in the ovipositor of *E. sophia* compared to the antennae. The full-length ORF (open reading frame) sequences of these genes were cloned and validated. The signal peptide was predicted using the SignalP 2.0 server (http://www.cbs.dtu.dk/services/SignalP-2.0/#submission). We designed cloning primers using Primer Premier 5.0 (Table S3). Polymerase chain reaction (PCR) reactions were performed using a 2× Phanta Max premix and a 25 μL system, following the provided instructions for maintaining PCR conditions. The amino acid sequences of OBPs were created and visualized using DNAMAN (LynnonBiosoft, USA). The amino acid sequences of OBPs from other insects used to construct the phylogenetic tree were downloaded from the NCBI database based on Blastx results (https://www.ncbi.nlm.nih.gov/). The maximum likelihood method in MEGA 7.0 was employed, using the Poisson model with complete deletion for handling gaps/missing data, and bootstrap=1000 (Tamura et al., 2013). The phylogenetic tree visualization was implemented using iTOL v6.0 (Letunic and Bork, 2024).

2.8 Tissue-specific expression analysis of candidate OBP genes

We collected ovipositors (4000 individuals per sample), antennae (including heads) (400 pairs per sample) and the remaining parts after collecting the above two tissues (400 individuals per sample) by dissecting adult female *E. sophia*. Each sample was studied with three biological replicates. All samples were immediately frozen in liquid nitrogen and stored at -80°C until use. Gene expression profiles of the preliminarily screened OBPs genes were analyzed. Specific primers were designed using Primer Premier 5.0 (Table S4). Total RNA was extracted using Trizol(Invitrogen, Carlsbad, CA, USA). The purity of RNA samples was checked by the absorbance ratios of A260/A280 and A260/A230, integrity was verified by electrophoresis on a 1.0% agarose gel, and concentration was determined using Nanodrop One (Thermo Fisher Scientific, Waltham, MA, USA). cDNA libraries were prepared using the Hifair III First Strand cDNA Synthesis SuperMix (Yeasen, Shanghai, China) for qPCR according to the manufacturer's instructions. qRT-PCR was performed using SYBR Green Master Mix (Yeasen Biotech, Shanghai, China) on an Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Each reaction was performed in a total volume of 20 μL, containing 1 μL template cDNA, 10 μL SYBR Green pre-mix, 0.4 μL of each primer (10 μM), and 8.2 μL ddH2O. PCR was run with a program of 30 s at 95°C, followed by 40 cycles of 95°C for 30 s and 60°C for 34 s. A melting curve was constructed at 95°C for 60 s. The amplification efficiency for each gene was optimized to maximize the peak throughout the amplification process. Each sample was performed with three biological replicates and three technical replicates. The *β-actin* gene was used as the reference gene for normalization, and the relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Results are presented as means (n = 3) ± standard error; significant

differences in expression levels were determined by One Way ANOVA, and plotted using GraphPad Prism 8.0.

2.9 Expression of candidate *EsopOBPs* in the ovipositor of females

DIG-labeled antisense RNA probes were synthesized using the DIG RNA Labeling Mix (Roche) kit. The Whole Mount Fluorescence In Situ Hybridization (WM-FISH) procedure followed the protocols outlined by Schultze et al. (2013) and Pregitzer et al. (2019). In detail, adult female *E. sophia* ovipositor were dissected and transferred to fixation solution (4% paraformaldehyde in 0.1 M $NaCO_3$, pH 9.5, 0.03% Triton X-100) for fixation at 6°C for 20-24 hours. After rinsing with PBS (phosphate-buffered saline = 0.85% NaCl, 1.4 mM KH_2PO_4, 8mM Na2HPO4, pH 7.1) containing 0.03% Triton X-100 at room temperature for 1 minute, the samples were incubated in 0.2 M HCl, 0.03% Triton X-100 for 10 minutes. Subsequently, the samples were washed three times in PBS containing 0.03% Triton X-100 for 3 minutes each, then transferred to the hybridization solution (containing 50% formamide, 5×SSC, 1×Denhardt's reagent, 50 μg/ml yeast RNA, 1% Tween 20, 0.1% Chaps, and 5 mM EDTA pH 8.0) for pre-hybridization at 55°C for 6 hours, followed by at least 48 hours of hybridization with the labeled *EsopOBP* antisense RNA probes at the same temperature. The samples were then washed four times at 60°C in 0.1xSSC, 0.03% Triton X-100 for 15 minutes each. After blocking with 1% blocking reagent (Roche) in TBS (100 mM Tris, 150 mM NaCl, pH 7.5) with 0.03% Triton X-100 for 5-6 hours, detection of DIG-labeled probes was performed by incubating with anti-DIG AP-conjugated antibody (Roche) diluted 1:500 in TBS, 0.03% Triton X-100 with 1% blocking reagent for at least 48 hours. Following five washes at room temperature in TBS with 0.05% Tween 20 for 10 minutes each, the samples were incubated at 6°C in the dark for 7-8 hours with HNPP (2-hydroxy-3-naphtoic acid-2'-phenylanilide phosphate, Roche) in DAP buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 8.0) to visualize the hybridization of DIG-labeled probes. After brief rinsing in PBS, samples were mounted in Mowiol (10% polyvinylalcohol 4-88, 20% glycerol in PBS), analyzed using a Zeiss LSM 980 laser scanning microscope (Zeiss, Oberkochen, Germany), and images were processed using ZEN 2012 software.

2.10 Expression and purification of *EsopOBP1* and *EsopOBP10*

Recombinant protein expression was carried out using the Escherichia coli expression system with an N-His tag. The full coding region lacking signal peptide sequences of *EsopOBP1* and *EsopOBP10* was subcloned into the NdeI/EcoRI restriction sites of the dephosphorylated pET-28b expression vector. Subsequently, the constructed plasmids were transformed into Escherichia coli BL21 (DE3) competent cells for further expression. The size and purity of the target proteins were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein blotting. The proteins were stored at -80°C for subsequent fluorescence competitive binding assays.

The fluorescence competitive binding assay was conducted using an F96 black ELIAS plate (Xinyou Biotechnology, Hangzhou, China) on a Synergy4 microplate reader (BioTek Instruments, Winooski, VT, USA). Probe 1-NPN and test ligands were dissolved in spectrophotometric methanol to prepare a 1.0 mM stock solution. Fluorescence probe 1-NPN was excited at 337 nm, and the emission spectrum was recorded between 390-490 nm. Initially, to determine the binding constants of 1-NPN with *EsopOBP1* and *EsopOBP10*, a 2.0 μM protein solution in 50 mM Tris-HCl (pH = 7.4) was titrated with 1 mM 1-NPN to achieve different concentrations. Subsequently, using 1-NPN as the fluorescence reporter and odorants as competitors, the competitive binding of specific compounds in the screened secondary hosts was examined. The concentration of protein and 1-NPN was maintained at 2.0 μM each; after 2 minutes of incubation of protein and 1-NPN in the wells of the ELIAS plate, odorants were added. The final concentrations of each competitor ranged from 2 to 20 μM. After the addition of odorants for 2 minutes, fluorescence intensity was measured and recorded. The total volume of the mixed solution in each well was kept at 250 μL. Each interaction was performed in triplicate.

2.11 Homology modeling and molecular docking of *EsopOBP1* and *EsopOBP10*

The three-dimensional (3D) models of *EsopOBPs* were constructed using AlphaFold2, and the predicted structural quality was evaluated using the SAVES server6 (https://saves.mbi.ucla.edu/). The two-dimensional (2D) structures of compounds with binding ability, used in fluorescence competition binding assays, were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and processed into 3D structures using Open-Babel v3.1.1. Molecular docking was then performed using AutoDock v4.2.6, and the docking results were visualized using PyMOL v2.4.0.

2.12 Quantification and statistical analysis

Quantitative and statistical methods are described in the figure legends and method details. Results are always expressed as mean ± standard error (SEM). Data were analyzed and plotted using SPSS 22.0 and GraphPad Prism 9.5.1. For significance testing, data conforming to or transformed to normal distribution were analyzed using independent samples t-tests for two-group comparisons, such as the analysis of behavioral time differences between *E. sophia* females during the process of producing female and male offspring (Table 1). For comparisons involving more than two groups, one-way ANOVA with Tukey's HSD was used, such as the analysis of *EsopOBPs* gene expression in different tissues of females (Figure 3A) and the statistical analysis of probing behavior of female wasps after treatment with different compound combinations (Figure 5). For data that did not conform to normal distribution after transformation, the Mann-Whitney test was used for two-group comparisons, and the Kruskal-Wallis test was used for comparisons involving more than two groups.

3. Results

3.1 Ovipositor role of *Encarsia sophia* females in their decision-making behavior for laying female and male offspring

To clarify the decision-making process for laying female and male eggs, we first observed the oviposition behavior of *E. sophia* when laying eggs on primary hosts (female eggs) and secondary hosts (male eggs) and recorded the duration of each behavioral phase. Both female and male oviposition behaviors consist of four stages (Figure S5-1). However, regardless of whether the female ultimately accepted the host, the internal probing duration of the ovipositor before laying eggs showed significant differences between female and male oviposition (Table S5-1, probing without laying eggs: $t = -3.66$, $df = 125.375$, $P < 0.001$; probing and laying eggs: $t = 5.58$, $df = 57$, $P < 0.001$). This suggests that the internal detection process plays an important role in the oviposition decision-making.

3.2 Receptor description from female *Encarsia sophia* ovipositor

The ovipositor of *E. sophia* consists of an ovipositor sheath (OS) and an ovipositor tube. The ovipositor tube is composed of two ventral valves (VV) and a dorsal valve (DV) with a semi-healed end. Seven types of sensilla were observed on the ovipositor, including physical mechanoreceptors such as Trichoid Sensilla, Böhm’ s bristles, and Slight Surface Depression, as well as chemical olfactory receptors such as Sensilla Campaniformia, Sensilla Basiconica, Sensilla Coeloclnica, and Dentate Sensilla (Figure 5-3).

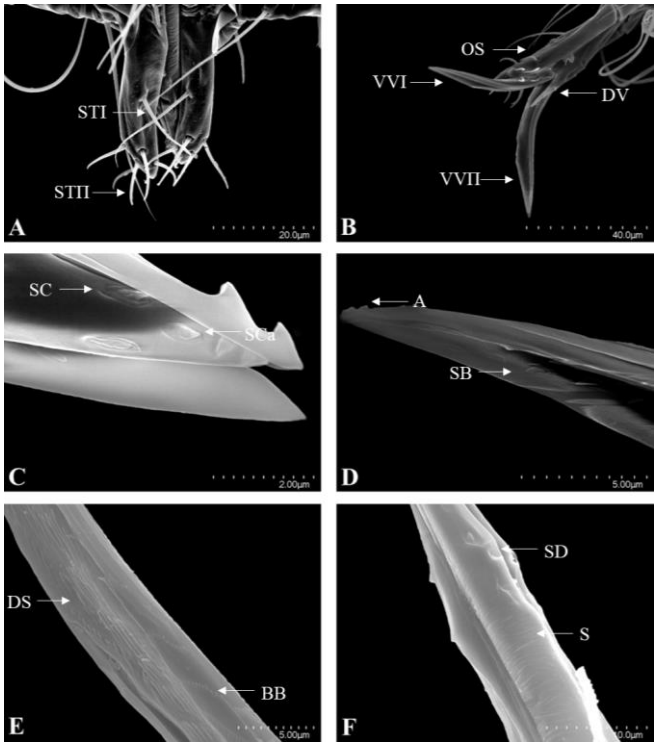


Figure 5-3. *Encarsia sophia* ovipositor sensilla

A, Ovipositor shape and surface of Trichoid Sensilla; B, Ovipositor Sheath, OS and Ventral Valves, VV + Dorsal Valves, DV; C, Sensilla Coeloclnica on the oviposition valve SC, Sensilla Campaniformia, SCa; D, Sensilla Basiconica, SB, Batb－like apophyses, A; E, Dentate Sensilla, DS, Böhm bristles, BB; F, Slight surface Depression, SD, Striation.

3.3 Factor assessment for *Encarsia sophia* female or male offspring production

The physical differences between primary and secondary hosts are mainly mechanical stress. To clarify the impact of host mechanical pressure on *E. sophia* female oviposition, *B. tabaci* nymphs (primary hosts: Figure 5-4A1, A2) and *E. hayati*-parasitized hosts at different developmental stages (secondary hosts: Figure 2B1, B2, B3) were provided with varying fluid states. Hosts in different states could all receive sex-specific eggs, indicating that the fluid quantity inside the host does not influence oviposition, and physical factors are not the main influencing factors.

However, for conspecific secondary hosts, eggs could only be laid at specific prepupal stages, not during the larval stage. The most noticeable difference between these stages is the amount of liquid inside the host (Figure 5-4C1, C2). We dissected conspecific secondary hosts and *E. hayati* at the same developmental stage and made an interesting discovery: the larvae within the conspecific hosts were highly active,

continuously wriggling when touched (Figure 5-4D, S5-2), whereas *E. hayati* larvae remained spherical and motionless (Figure 5-4E). We hypothesized that the activity level of conspecific larvae affects oviposition, rather than the dry state of the host. To reduce the activity of conspecific larvae, we refrigerated them at 4°C for 2 hours before presenting them to unmated *E. sophia* females. The oviposition rate on these hosts increased from 0% (untreated) to 37%. Therefore, we not only demonstrated that physical factors are not the primary influence on oviposition but also identified the true reason why conspecific larvae cannot be oviposited upon during their larval stage. This may be a self-protection strategy used by *E. sophia* to reduce intraspecific competition.

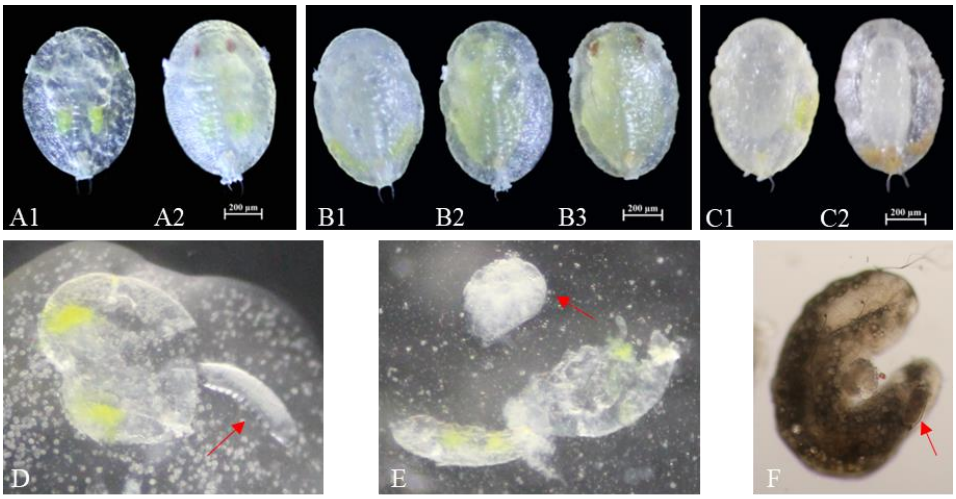


Figure 5-4. The impact of mechanical pressure from hosts on *Encarsia sophia* oviposition. Nymphs of different ages of *B. tabaci* (primary hosts): 2nd instar (A1), 4th instar (A2); different developmental stages of *E. hayati* (secondary hosts): larvae (B1), prepupae (B2), pupae (B3); different developmental stages of *E. sophia* (conspecific secondary hosts): larvae (C1), prepupae (C2); dissected conspecific secondary host larvae of *E. sophia* (D, arrow indicating *E. sophia* larva inside *B. tabaci*); dissected heterospecific secondary host larvae of *E. hayati* (E, arrow indicating *E. hayati* larva inside *B. tabaci*); dissected image of secondary host larvae of *E. sophia* after cold treatment and oviposition of male eggs, captured under transmitted light using an Olympus BX41 microscope (F, arrow indicating *E. sophia* male egg).

3.4 Male egg induction in *Encarsia sophia* females by n-Heptacosane from secondary hosts

To clarify the chemical factors of the host affecting the oviposition of females, host compounds were first tested to determine their influence on the oviposition decisions of *E. sophia* females. i In hosts treated with the 5-minute extract, the male egg production rate was 3.3% (Table S5-2), while the same hosts in the untreated control

group and the solvent n-hexane-treated control group had an oviposition rate of 0 (n > 200). This indicated that chemical factors influenced the oviposition judgment of the females, and that the 5-minute extract contained effective male-inducing compounds that prompted the wasps to lay male eggs in hosts where female eggs would normally be laid.

We compared and screened 12 compounds that were common among 3-4 secondary hosts but absent in the primary host (Table 5-1, Figure S5-3). These secondary host-specific volatiles were then diluted in n-hexane at four concentration gradients and applied to the primary host (*B. tabaci* nymph) which cannot be used for male egg production. Unmated *E. sophia* females (which can only produce male eggs) were provided with these treated hosts for 2 hours, after which the hosts were dissected and the oviposition rates were counted. Surprisingly, it was found that hosts treated with 10 μg/mL of Heptacosane had a male egg production rate of 10% (Table 5-2), which was higher than the oviposition rate induced by the extract. Interestingly, a unique situation was observed where the females laid male eggs on the outer surface of the host's shell when the primary host, *B. tabaci* nymphs, were treated with Heptacosane. Previously, whether laying female or male eggs, the females always deposited them inside the host's shell.

Table 5-1. In the host extract of n-hexane for 5 minutes, unique compounds in the secondary host relative to the primary host(n=6).

	Chemical formula	Compounds	CAS
1	$C_{19}H_{40}$	Nonadecane	629-92-5
2	$C_{20}H_{42}$	Eicosane	112-95-8
3	$C_{27}H_{56}$	Heptacosane	593-49-7
4	$C_{21}H_{44}$	Heneicosane	629-94-7
5	$C_{9}H_{20}$	2,4-Dimethylheptane	2213-23-2
6	$C_{16}H_{22}O_{4}$	Dibutyl phthalate	84-74-2
7	$C_{34}H_{58}O_{4}$	Ditridecyl phthalate	119-06-2
8	$C_{15}H_{30}O$	Pentadecanal	2765-11-9
9	$C_{44}H_{88}O_{2}$	Docosyl docosanoate	17671-27-1
10	$C_{31}H_{64}$	Hentriacontane	630-04-6
11	$C_{36}H_{74}$	Hexatriacontane	630-06-8
12	$C_{16}H_{32}O$	Hexadecanal	629-80-1

Table 5-2. Male egg laying rate of *Encarsia sophia* virgin females after adding different concentrations of compounds to *Bemisia tabaci* nymph.

Compounds	The oviposition rate under different concentration treatments %			
	0.1	1	10	100 μg/mL
n-hexane	0	0	0	0
Nonadecane	0	0	0	0
Eicosane	0	0	0	0
Heptacosane	0	0	**10**	0
Heneicosane	0	0	0	0
2,4-Dimethylheptane	0	0	0	0
Dibutyl phthalate	0	0	0	0
Ditridecyl phthalate	0	0	0	0
Pentadecanal	0	0	0	0
Docosyl docosanoate	0	0	0	0
Hentriacontane	0	0	0	0
Hexatriacontane	0	0	0	0
Hexadecanal	0	0	0	0

3.5 Identification of odorant-binding protein genes in the ovipositor of *Encarsia sophia*

To reveal the molecular mechanisms underlying the female and male egg production decisions of *E. sophia* in their respective hosts, we conducted transcriptomic sampling and sequencing analysis of the female's antennae and ovipositor. (Figure 5-5A) and (Figure 5-5B). Sequencing was conducted on six cDNA libraries (three from female antennae and three from female ovipositors), resulting in a total of 270,223,666 raw reads. After trimming and cleaning, 263,739,334 clean reads were obtained, with a Q20 value exceeding 96.43% for each sample. The GC content ranged from 34.57% to 39.34%. A total of 19,063 annotated genes were obtained. Additionally, 4149 new genes were annotated in the unannotated transcript regions of the genome, as identified in the Pfam database.

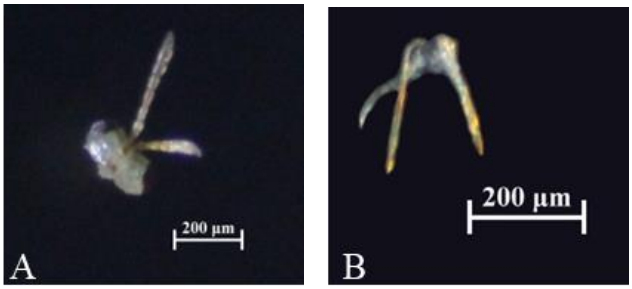
The analysis of differential gene expression in the transcriptomes of *E. sophia* antennae and ovipositors revealed a total of 5649 differentially expressed genes (DEGs), comprising 2593 upregulated genes and 3056 downregulated genes (Figure S5-4A). Hierarchical clustering analysis of the DEGs showed a uniform distribution, indicating significant differences in gene expression between the antennae and ovipositors of *E. sophia* (Figure S5-4B). GO functional enrichment analysis was

performed on the DEG set, and the top 30 most significant terms were selected for visualization in a bar graph, categorized into biological process, cellular component, and molecular function. Among them, 77 DEGs were annotated as odorant binding genes (Figure S5-4C). KEGG pathway enrichment analysis, with a threshold of padj less than 0.05 for significance, selected the top 20 significantly enriched KEGG pathways for visualization (Figure S5-4D).

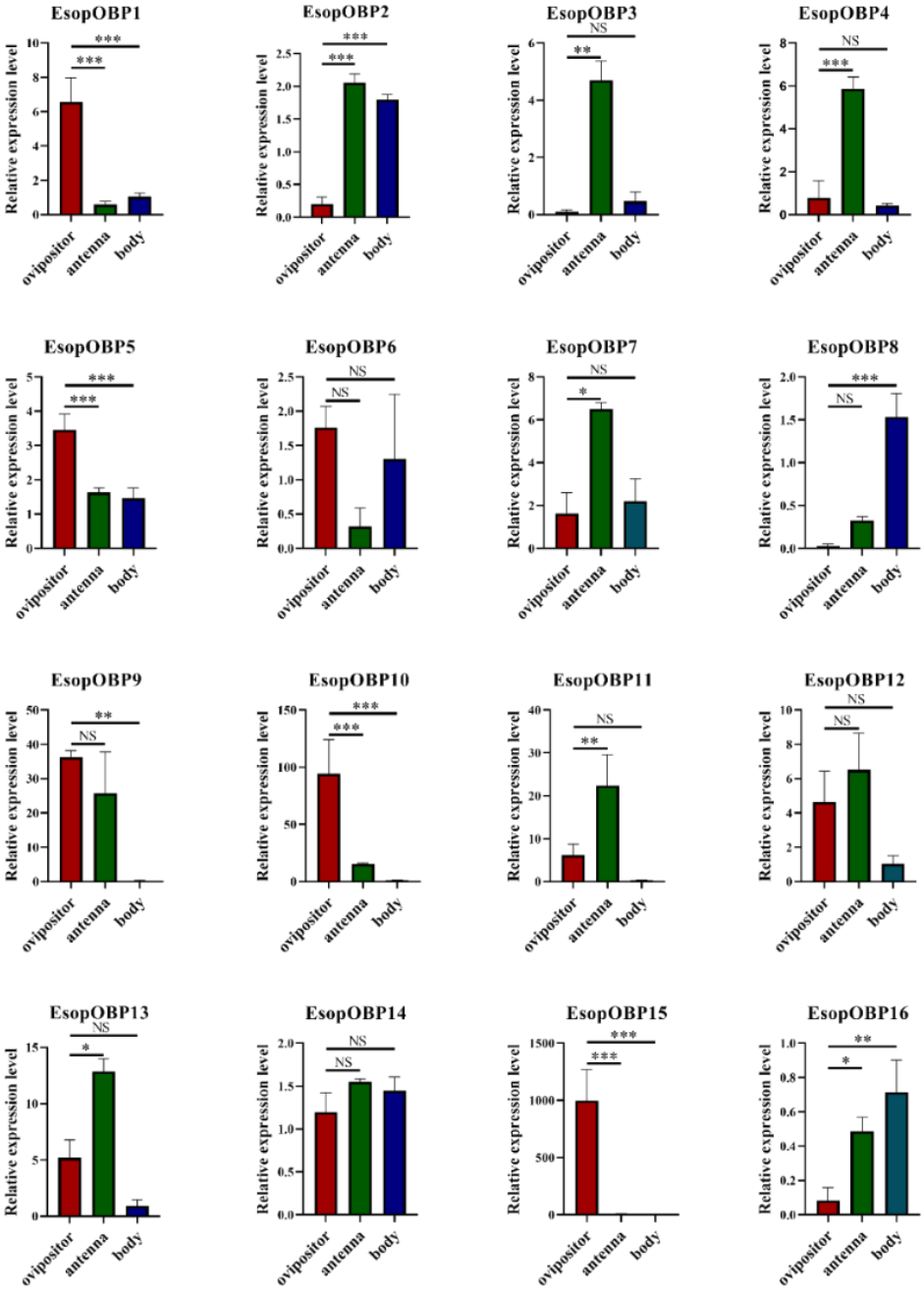
To further identify the olfactory genes that bind to the target compounds, we found that among the aforementioned differentially expressed genes, 16 odorant-binding proteins (OBPs) had significantly higher expression levels in the ovipositor compared to the antennae (Figure S5A). The cDNA of the identified 16 OBPs was cloned, all of which had complete open reading frames (ORFs) encoding 122 to 169 amino acids, and all had signal peptides, with N-terminal signal peptide sequences ranging from 16 to 24 amino acids. 9 (EsopOBP7, 8, 9, 10, 11, 12, 14, 15, and 16) belonged to Classic-C OBPs, while the other 7 (EsopOBP1, 2, 3, 4, 5, 6, and 13) belonged to Minus-C OBPs (Figure S5B). BLAST searches against the NCBI database showed significant similarity of the candidate OBPs' amino acid sequences with those of other Hymenoptera species, such as *E. formosa*, *N. vitripennis*, *Copidosoma floridanum*, *Chouioia cunea*, *T. dendrolimi*, *L. heterotoma*, and *Fopius arisanus*. Phylogenetic trees were constructed using the identified 16 OBP sequences from the ovipositor of *E. sophia* and OBPs from other Hymenoptera species, indicating clustering of candidate OBPs from *E. sophia* ovipositors with those from other species in the phylogenetic analysis (Figure S5-5C).

To clarify the expression patterns of the candidate genes in different parts of the female *E. sophia*, tissue expression analysis of the 16 candidate OBPs identified in the ovipositor of *E. sophia* was conducted using qRT-PCR in the antennae (including the head), ovipositor, and other body parts of females. The expression levels of *EsopOBP1*, *EsopOBP5*, *EsopOBP10*, and *EsopOBP15* were significantly higher in the ovipositor compared to other tissues (Figure 5-5C). This indicates that they may have specific functions on the ovipositor.

To localize and identify the cellular expression of the *EsopOBP* genes with specific expression in the ovipositor of *E. sophia*, we employed the Whole Mount Fluorescence In Situ Hybridization (WM-FISH) method using specific RNA probes targeting the ovipositor. When experiments were conducted using digoxigenin (DIG)-labeled probes specific to *EsopOBP1* and *EsopOBP10*, cells marked in red were observed on the ovipositor (Figure 5-5D). Interestingly, comparison with the scanning electron microscopy results of the ovipositor in Figure 1 revealed that *EsopOBP1* and *EsopOBP10* were localized to chemosensory structures. Therefore, it is speculated that these two genes may be associated with olfactory perception during oviposition in females, warranting further functional exploration.


200 µm
A
200 µm
B

C


EsopOBP1
EsopOBP2
EsopOBP3
EsopOBP4
EsopOBP5
EsopOBP6
EsopOBP7
EsopOBP8
EsopOBP9
EsopOBP10
EsopOBP11
EsopOBP12
EsopOBP13
EsopOBP14
EsopOBP15
EsopOBP16
Relative expression level
ovipositor
antenna
body
NS
*
**
***

D

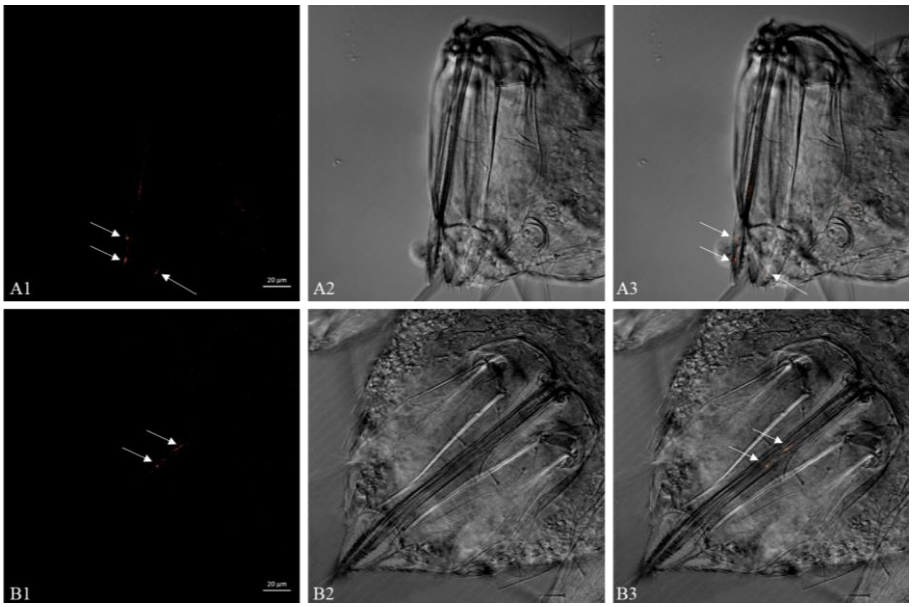


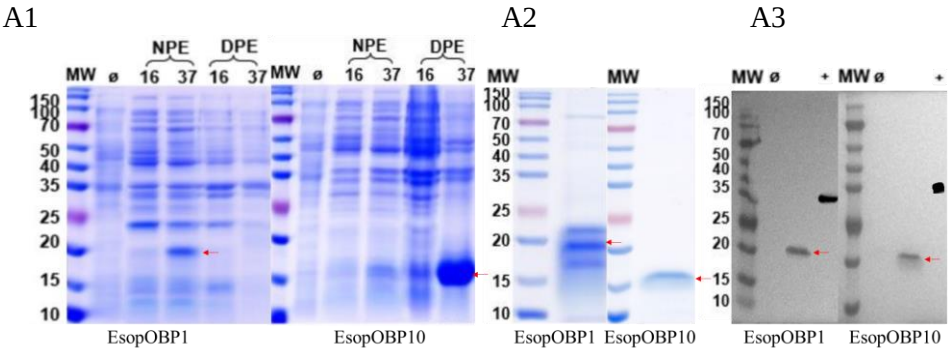
Figure 5-5. Identification of candidate odorant-binding protein OBPs in the ovipositor of *Encarsia sophia*. (A) Antennae sampling (including the head). (B) Ovipositor sampling. (C) The relative expression levels of candidate OBPs genes in different tissues of adult female *E. sophia* were detected using qRT-PCR. The expression data was normalized to the β-actin gene using the 2^−ΔΔCt method. Data are presented as mean ± SEM (n=3). Asterisks indicate statistically significant differences determined by one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, NS indicates no significant difference. (D) Expression localization of OBPs in *E. sophia* ovipositor. (A1-A3: *EsopOBP1* B1-B3: *EsopOBP10*) A1-B1: Expression positions of *EsopOBP1* and *EsopOBP10* under dark field; A2-B2: Control under bright field; A3-B3: Expression positions of *EsopOBP1* and *EsopOBP10* under bright field after superposition.

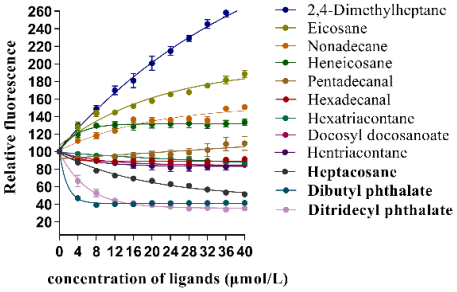
3.6 *EsopOBP1* and *EsopOBP10* binding affinity with the secondary host-specific compounds

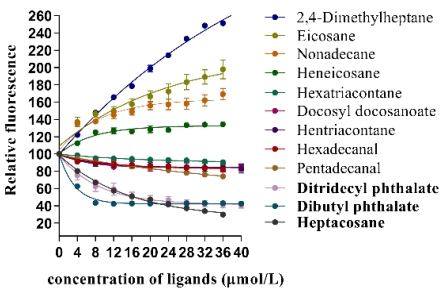
To determine whether the candidate genes *EsopOBP1* and *EsopOBP10* are involved in the detection of host compounds, we first expressed the recombinant proteins of *EsopOBP1* and *EsopOBP10* in a prokaryotic expression system (Figure 5-6A). *EsopOBPs* exhibited linear Scatchard plots and typical saturation binding curves with 1-NPN. The dissociation constants (Kd) of *EsopOBP1* and *EsopOBP10* with 1-NPN were determined to be 20.66 and 14.14 μmol/L, respectively, indicating that 1-NPN serves as a suitable fluorescent probe for ligand binding characteristics of these two OBPs (Figure S5-6). Competitive fluorescence binding assays demonstrated that *EsopOBP1* and *EsopOBP10* exhibited strong binding affinity with Heptacosane, Dibutyl phthalate, and Ditridecyl phthalate, reducing fluorescence intensity to below

50% of the initial value (with dissociation constants Ki of 15.43, 7.62, 9.78 and 14.54, 10.56, 17.47 μM, respectively). They showed no significant binding affinity with the other compounds tested, which is consistent with the results of our earlier oviposition induction behavioral experiments where Heptacosane exhibited effects (Figure 5-6B).

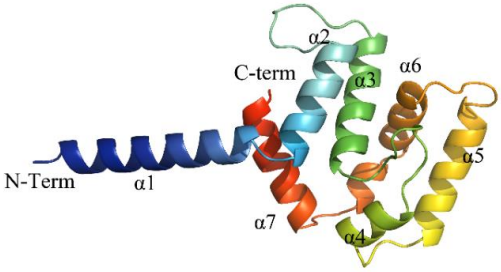
To further understand the molecular interactions between *EsopOBPs* proteins and their ligands, we constructed their three-dimensional models using homology modeling and performed molecular docking simulations. The homology-based 3D model of *EsopOBP1* protein features seven α-helices (α1−α7) along with N-terminal and C-terminal ends, while the *EsopOBP10* protein model has six α-helices (α1−α6) along with N-terminal and C-terminal ends (Figure 5-6C). The 3D structural models were evaluated using a Ramachandran plot, confirming the reliability of the constructed *EsopOBPs* protein models (Figure S5-7). Secondary host-specific volatiles were docked into the binding pockets of *EsopOBP1* and *EsopOBP10*. Molecular docking was performed to identify key amino acids involved in the interactions. For the ligand Heptacosane, the binding energy with *EsopOBP1* was -4.9 kcal/mol, forming hydrophobic interactions with ILE50, ASN53, TYR33, THR38, GLU34, ARG30, ILE137, LYS40, VAL39, and LEU45. The binding energy with *EsopOBP10* was -5.0 kcal/mol, forming hydrophobic interactions with PRO47, PHE57, LEU58, TYR118, GLN127, TYR81, LEU129, and ILE43 (Figure 5-6D). For the ligand Dibutyl phthalate, the binding energy with *EsopOBP1* was -4.6 kcal/mol, forming hydrophobic interactions with TYR33, GLU34, ARG30, THR38, and a salt bridge interaction with LYS40. The binding energy with *EsopOBP10* was -6.1 kcal/mol, forming hydrophobic interactions with MET61, LEU58, TYR118, LEU129, a hydrogen bond interaction with GLN127, and a salt bridge interaction with LYS67 (Figure 5-6E). For the ligand Ditridecyl phthalate, the binding energy with *EsopOBP1* was -5.3 kcal/mol, forming hydrophobic interactions with LEU45, VAL39, THR38, LYS40, VAL136, ARG30, GLU34, TYR33, and ILE50. The binding energy with *EsopOBP10* was -6.2 kcal/mol, forming hydrophobic interactions with LEU58, PHE57, TYR118, ILE43, LEU129, GLN127, ILE86, HIS85, TYR81, and a π-cation interaction with LYS67 (Figure 5-6F).



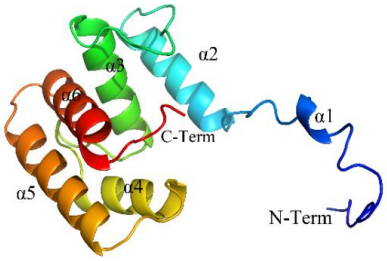




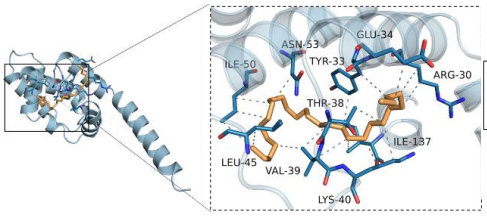
C1



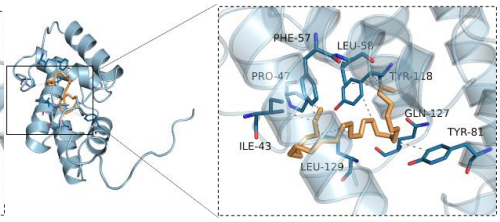
C2



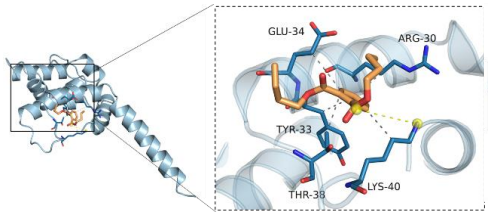
D1



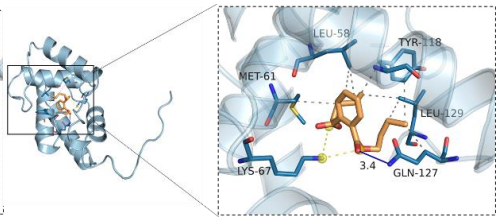
D2



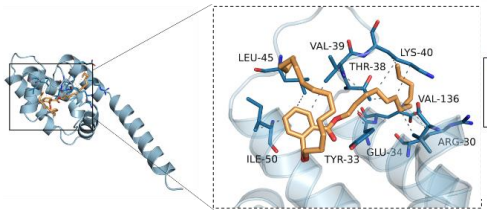
E1



E2



F1



F2

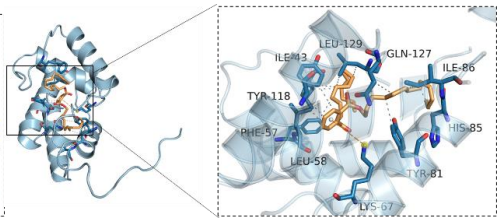


Figure 5-6. Recombinant *EsopOBP* protein stability with ligands and its homologous modeling and molecular docking. (A) Induction and purification of recombinant proteins *EsopOBP1* and *EsopOBP10*, (A1) Induction expression with 1 mM IPTG at 16°C/16h and 37°C/4h, respectively; (A2) Analysis of SDS-PAGE of protein final samples; (A3) Western blot of proteins. Molecular weight marker (M), from top to bottom: 150, 100, 70, 50, 40, 35, 25, 20, 15, 10 kDa. NPE: supernatant, DPE: inclusion bodies, Ø: negative control, +: positive control. (B) Binding curves of *EsopOBP1*(B1) and *EsopOBP10*(B2) proteins with different ligands. (C) 3D Structures of *EsopOBP1* and *EsopOBP10* (C1, C2); (D) Binding Modes and Key Residues of Heptacosane with *EsopOBP1* and *EsopOBP10* (D1, D2); (E) Binding Modes and Key Residues of Dibutyl Phthalate with *EsopOBP1* and *EsopOBP10* (E1, E2); (F) Binding Modes and Key Residues of Ditridecyl Phthalate with *EsopOBP1* and *EsopOBP10* (F1, F2).

3.7 Role of Heptacosane in the probing frequency of female *Encarsia sophia* towards the host

Based on the fluorescence competition experiments and molecular docking results of *EsopOBPs* with secondary host volatiles, compounds with binding affinity, including Heptacosane, Dibutyl phthalate, and Ditridecyl phthalate, were selected to investigate the effect of their mixture on the oviposition behavior of female *E. sophia*. Using the area normalization method by GC-MS, the compounds were mixed according to their peak area ratio (Heptacosane:Dibutyl phthalate:Ditridecyl phthalate = 60:16:5). The mixture was then applied to *B. tabaci* nymphs, with individual compounds used as controls. The probing behavior of virgin female wasps towards the host was observed and recorded within 1 hour, and the oviposition rate was determined by dissecting the hosts. Excepting for the Heptacosane treatment, which induced the females to lay male eggs in the *B. tabaci* nymphs, no oviposition was observed in the other treatments. Additionally, the probing frequency of the females towards the host was significantly increased in the mixture containing Heptacosane compared to the single compounds Dibutyl phthalate and Ditridecyl phthalate (Figure 5-7). These results further confirmed the important role of Heptacosane in inducing male egg production in *E. sophia*.

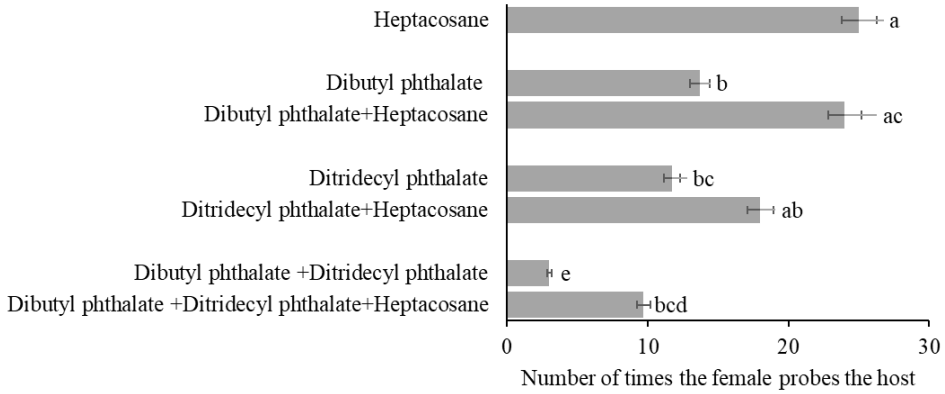


Figure 5-7. The behavior of *Encarsia sophia* females in producing male eggs within 1 hour after the addition of different combinations of compounds to *Bemisia tabaci* nymphs. Different letters indicate significant differences at the $P < 0.05$ level by one-way analysis ANOVA.

4. Discussion

In this study, we revealed the mechanism of heteronomous oviposition in the special biological control agent, the heteronomous hyperparasitoid *E. sophia*. Females use olfactory genes on their ovipositors to differentiate between various types of hosts. By screening for differential compounds in four different types of secondary hosts (used for producing male offspring) and a specific primary host (used for producing female offspring), we identified the compound n-heptacosane, which is unique to secondary hosts and induces oviposition for male eggs. Additionally, we found that the odorant-binding proteins *EsopOBP1* and *EsopOBP10* on the female's ovipositor detect this compound.

After locating the host habitat from a long distance, parasitoids typically use their antennae or ovipositors to assess host suitability to ensure they lay eggs in an appropriate host.[3] Parasitoids such as *T. chilonis*, *A. rhopalosiphi*, and *C. flavipes* exhibit host recognition and acceptance by touching the host surface with their antennae (Obonyo et al., 2010a,b). Parasitoids like *Leptopilina heterotoma* and *Diachasmimorpha longicaudata* use their ovipositors to sense unparasitized and parasitized hosts (Van, 1981; Montoya et al., 2003). The heteronomous hyperparasitoid has a unique oviposition strategy, laying female eggs in primary hosts and male eggs in secondary hosts, with different host relationships for male and female offspring (Yang et al., 2012). We observed and compared the oviposition behavior of *E. sophia* on primary and secondary hosts. Similar to other primary parasitoids (Zhang et al., 2021), the oviposition process includes antennal probing of the host surface, ovipositor insertion to detect the host, oviposition, and departure. Interestingly, there was no time difference in external host detection for laying female or male eggs, which may be related to the external shell of *B. tabaci*. However, significant differences were observed in ovipositor probing time for internal detection of primary and secondary hosts, regardless of whether the female eventually accepted the host for oviposition. Therefore, we hypothesize that *E. sophia* relies on ovipositor insertion to differentiate between hosts for laying female and male eggs. We then used scanning electron microscopy to observe the structure and receptors of the female's ovipositor. The ovipositor has both physical receptors, such as Trichoid Sensilla, Böhm's bristles, and slight surfaces (Ochieng et al., 2000; Ahmed et al., 2013; Huang et al., 2018), and chemical receptors, such as Sensilla Campaniformia, Sensilla Basiconica, Dentate Sensilla, and Sensilla Coeloconica (Bleeker et al., 2004; Goubault et al., 2011; Shah, 2012). Our results indicate that the ovipositor of heteronomous parasitoids has well-developed receptors. Therefore, we investigated the oviposition mechanism from both physical and chemical perspectives.

The physical factors influencing oviposition decisions of parasitoids mainly include temperature and humidity, light, host shape, and internal pressure (Godfray, 1994).

For the primary and secondary hosts utilized by heteronomous hyperparasitoids, the factor is the difference in internal pressure of the hosts. Previous reports suggested that *E. sophia* and other heteronomous hyperparasitoids can only produce males in dry host environments, where the primary parasitoids must consume the host's body fluids to create a dry internal space (Gerling, 1983). However, our results indicate that the parasitoids can lay corresponding female/male eggs inside hosts regardless of the varying internal pressures of primary or secondary hosts. This not only contradicts previous views but also suggests that physical factors of the host are not the main factors for heteronomous hyperparasitoids in distinguishing hosts.

In addition to physical factors, it is generally believed that parasitoids use gustatory and olfactory cues to distinguish their hosts (Goubault et al., 2011). We found that the Sensilla Coeloconica and Sensilla Campaniformia on the ovipositor of *E. sophia* are typical chemical olfactory receptors associated with the parasitoids' ability to detect host odors at close range (Lv et al., 2020; Del et al., 2021). Heteronomous hyperparasitoids like *E. sophia* can utilize a single primary host but multiple secondary hosts (Hunter and Woolley, 2001). Considering that the compounds detected by the females during oviposition are contact volatile compounds, we used n-hexane extraction to screen for compounds present in various secondary hosts but absent in the primary host. We identified 4-12 such compounds and tested the crude extracts and various concentrations of these compounds for their ability to induce oviposition in female *E. sophia*. Our results showed that the compound n-heptacosane has a male-inducing effect, prompting the females to lay male eggs on non-corresponding hosts and even lay male eggs outside the host. Hydrocarbons are usually involved in insect communication (Chapman, 1998). In some parasitoid species, the chemicals used for host marking are believed to be mainly composed of hydrocarbons (Greany and Oatman, 1972; Guillot et al., 1974; Rosi et al., 2001). Studies have shown that the aphid parasitoid *Praon volucre* exhibits an avoidance response to n-heptacosane left by the predatory ladybird *Coccinella septempunctata* (Nakashima et al., 2006). Here, we have discovered for the first time that n-heptacosane also has an oviposition-inducing effect. In subsequent mixing experiments, it was similarly proven that n-heptacosane can enhance *E. sophia*'s probing response to the host. This finding provides the potential for heteronomous hyperparasitoids to directly lay male eggs on primary host target pests in biological control. Additionally, the females' response to the compound exhibits a dose-effect relationship, which could be the reason for different parasitism "windows" for different hosts, serving as a trade-off mechanism in the competition among parasitoids. *Drosophila melanogaster* larvae adjust their oviposition sites based on the dose of (Z)-9-octadecenoic acid ethyl ester (OE), where high doses of OE are repellent, while low doses are attractive (Zhang et al., 2023).

We identified the key odorant-binding protein genes, *EsopOBP1* and *EsopOBP10*, in the ovipositor of female *E. sophia*. Notably, this is the first time that functional genes have been identified in the ovipositor of Hymenoptera parasitoids. Previous reports on the functional genes of insect ovipositors have mostly focused on *Drosophila* fruit flies and Lepidoptera moths (Diamandi et al., 2024; Zhang et al.,

2024). The following evidence confirms the olfactory function of these genes related to oviposition decision-making in the ovipositor: 1) They are significantly expressed in the ovipositor compared to the antennae of females. 2) Fluorescent in situ hybridization shows expression in the olfactory receptors on the ovipositor. 3) They have a strong binding affinity to the oviposition-inducing compound, n-heptacosane. However, due to the extremely small size of this parasitoid wasp, the RNAi technology for this type of parasitoid has not yet been developed internationally, making it impossible to verify the phenotypic characteristics after gene interference. This will be one of our research directions in the future.

In conclusion, we have discovered that *E. sophia* females use *EsopOBP1* and *EsopOBP10* on their ovipositors to detect n-heptacosane in their hosts, thereby determining whether to lay female or male eggs. This discovery leads to compelling questions: Could it be possible to manipulate the sex ratio of parasitic natural enemies by altering the host's odor? Furthermore, could the impact of such olfactory cues on sex determination extend beyond this specific group, influencing a broader range of insect species? These questions open new avenues for exploration in the field of insect reproductive strategies.

5. Supplementary data

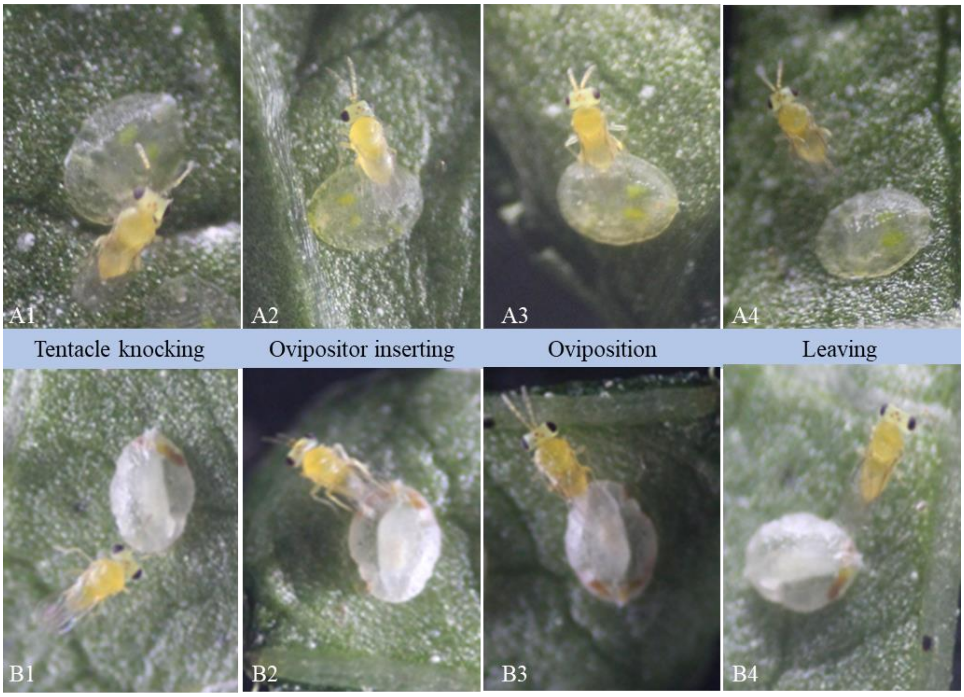


Figure S5-1. The behavioral process of *Encarsia sophia* when laying female eggs (on primary hosts: *Bemisia tabaci* nymphs, A) and male eggs (on secondary hosts: parasitized *Bemisia tabaci* nymphs, B) includes the following stages: antennal knocking (A1, B1), ovipositor inserting (A2, B2), oviposition (A3, B3), and leaving (A4, B4).

Table S5-1. Comparison of behavior time (Mean ± SEM) between producing female and male offspring of *Encarsia sophia* female

Mode of reproduction	External host examination time	Internal host examination time without oviposition	Internal host examination+ oviposition time
Producing female offspring	11.26 ± 0.81 a	120.90 ± 9.88 a	229.34 ± 15.31 b
Producing male offspring	10.06 ± 0.54 a	80.93 4.83 b	412.89±29.95 a

Note: Data followed by different lowercase letters in same column indicate significantly different at 0.05 level (t - text).

A video of the dissection of the larval stage of conspecific secondary host.MOV

Figure S5-2. A video of the dissection of the larval stage of conspecific secondary host (larval stage *Encarsia sophia*) in Phosphate-buffered saline (PBS).

Table S5-2. The male egg production rate of *Encarsia sophia* virgin female on primary hosts(n＞200).

Host	Ovipo-sited rate %	Oviposited rate after dripping n-hexane %	Oviposition rate after dripping crude extract of *A. gifuensis* larvae at different extraction times %				
			1	5	30	60	/min
Healthy *B. tabaci* nymph	0	0	0	**3.3**	0	0	

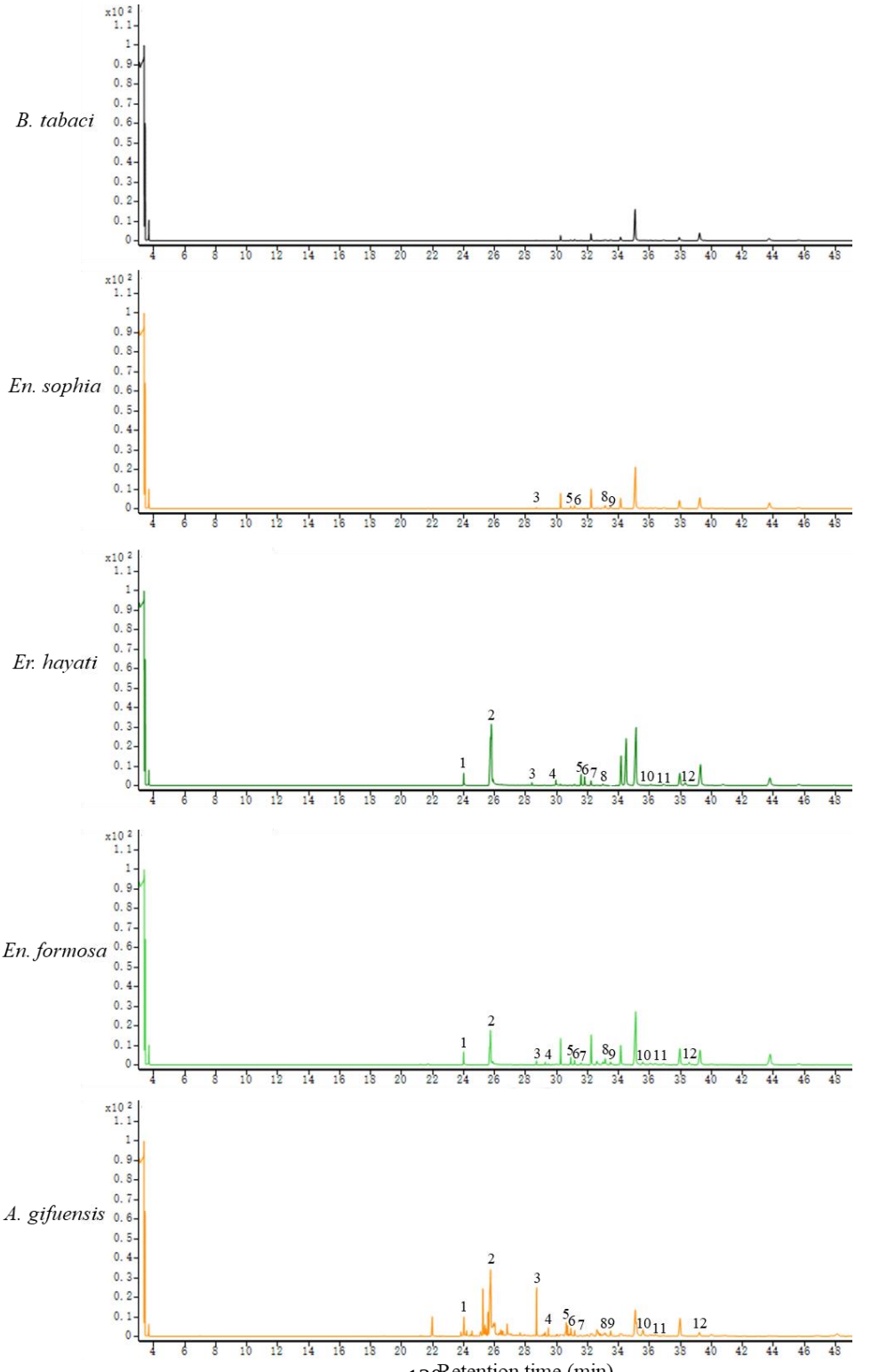

B. tabaci
En. sophia
Er. hayati
En. formosa
A. gifuensis
Retention time (min)

Figure S5-3. A representative GC-MS profile shows 5 minutes the hexane-extracted volatiles of *Encarsia sophia*'s primary host (*Bemisia tabaci*) and different types of secondary hosts (conspecific - *Encarsia sophia*, congeneric - *Encarsia formosa*, confamilial - *Eretmocerus hayati*, and heterofamilial - *Aphidius gifuensis*). For the identification of numbered peaks in the GC-MS chromatograms, refer to the methods section. Specific compounds correspond to the numbers listed in Table 1.

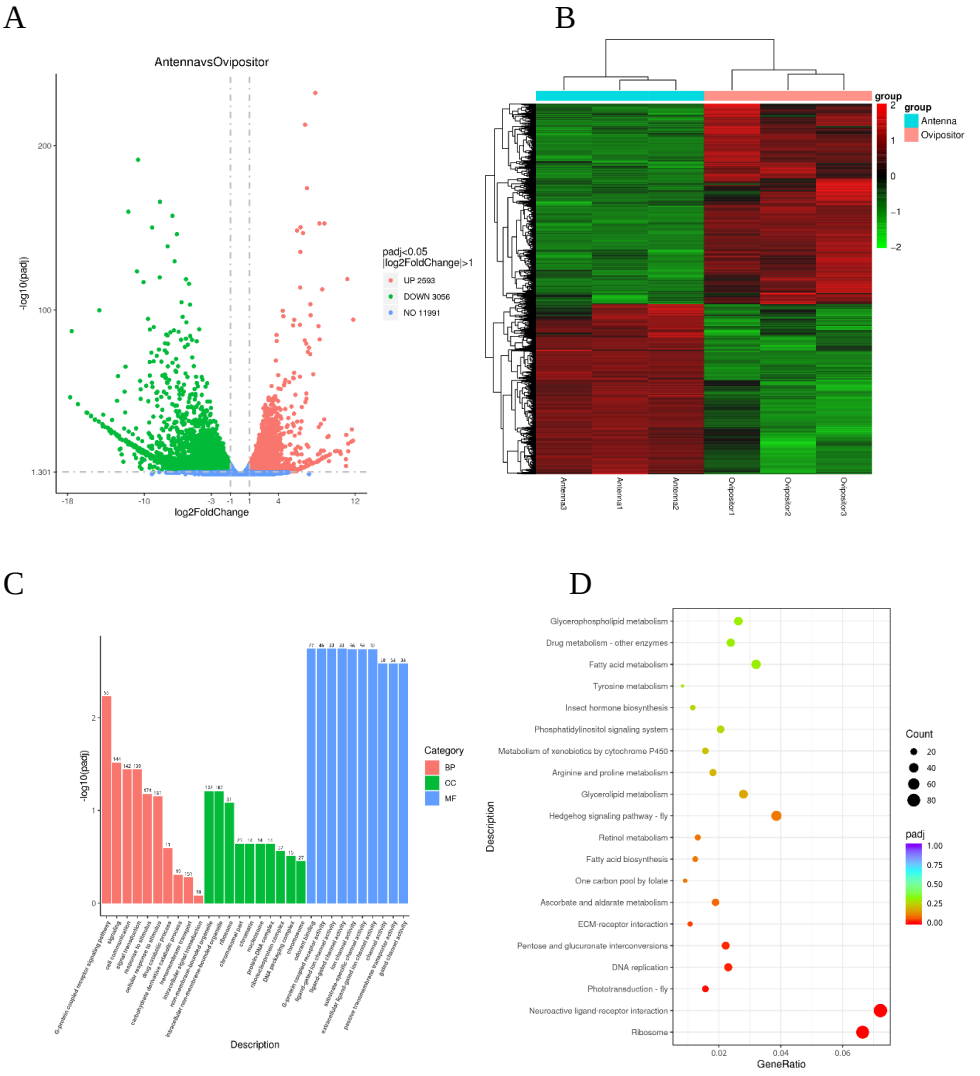
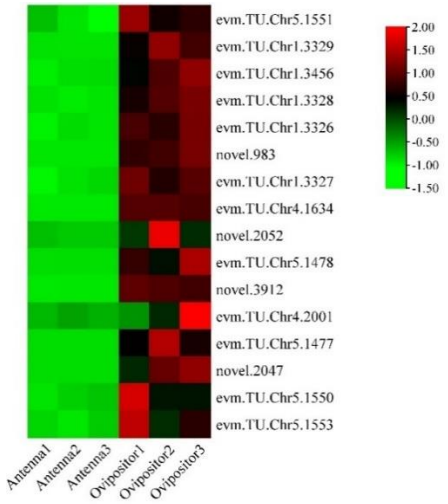


Figure S5-4. Transcriptome sampling and differential gene expression analysis of the antennae and ovipositor in *Encarsia sophia* females. (A) volcano plot of differentially expressed genes, with log2FoldChange values on the horizontal axis and -log10padj or -log10pvalue on the vertical axis. The blue dashed line represents the

threshold line for differential gene selection criteria. (B) heatmap of clustered differentially expressed genes, with sample names on the horizontal axis and normalized FPKM values of differentially expressed genes on the vertical axis. The color spectrum ranges from red indicating higher expression levels to green indicating lower expression levels. (C) bar graph of Gene Ontology (GO) enrichment analysis, with GO terms on the horizontal axis and the significance level of GO term enrichment represented by -log10(padj) on the vertical axis. Different colors represent different functional categories. (D) scatter plot of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, with the ratio of differentially expressed genes annotated to KEGG pathways to the total number of differentially expressed genes on the horizontal axis and KEGG pathways on the vertical axis.

A



B

C

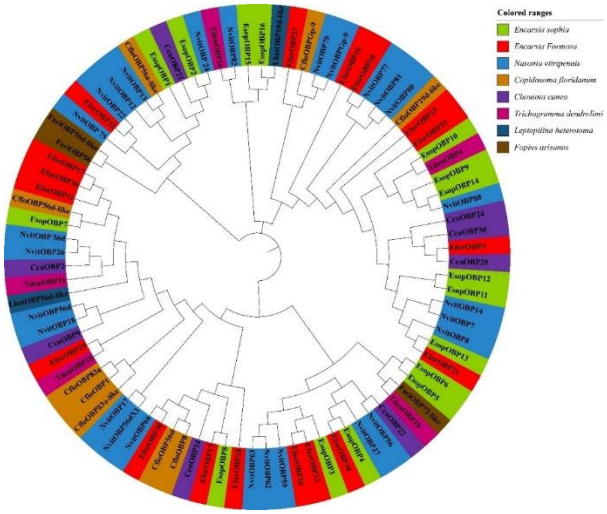


Figure S5-5. Identification of candidate OBPs in the ovipositor of *Encarsia sophia*. (A) OBPs significantly upregulated in the ovipositor, with sample names on the horizontal axis and normalized FPKM values of differentially expressed genes on the vertical axis. The color spectrum ranges from red indicating higher expression levels to green indicating lower expression levels. (B) Multiple sequence alignment of *EsopOBPs*. (C) Phylogenetic tree of candidate OBPs from *E. sophia* and OBPs from other Hymenoptera species based on amino acid sequences (constructed using maximum likelihood method, with 1000 repetitions).

A B

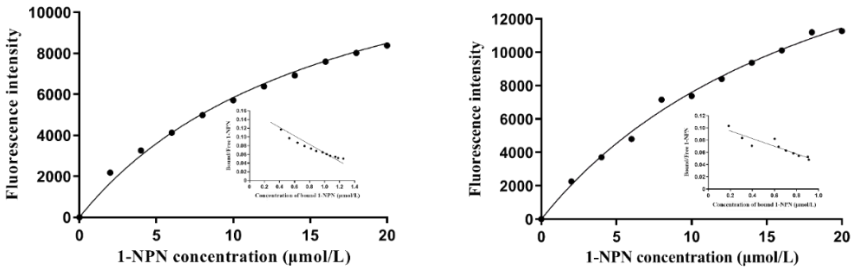


Figure S5-6. Binding curves of *EsopOBP1*(A) and *EsopOBP10*(B) proteins with 1-NPN and Scatchard linear transformation.

A **B**

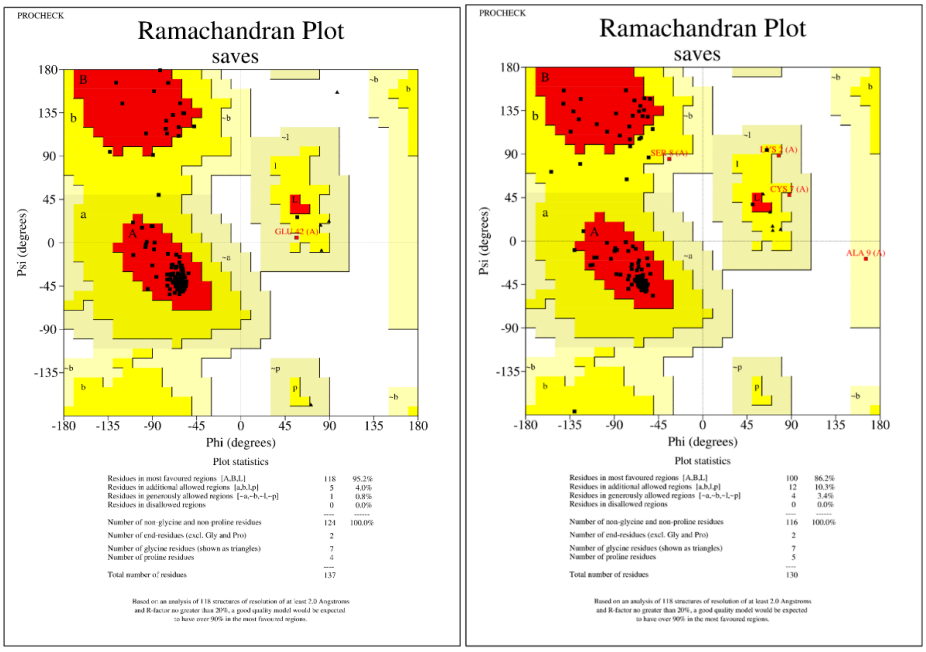


Figure S5-7. Ramachandran plots(A, *EsopOBP1*) and(B, *EsopOBP1*)

Table S5-3. Primers for *Encarsia sophia* OBP cloning

OBP	Forward primer (5'-3')	Reverse primer (5'-3')
EsopOBP1	ATGAAATTTGTCGGAG	ATGACCATGGAAAATG
EsopOBP2	AGCAAAGCATCATCCAAAA	CATGCAAATTGCGCTAATC
EsopOBP3	TGATAATCATCATGAATCT	TCTGTTTTATTGTAGTTTG
EsopOBP4	CTAAAAATGAGAGAAGCGT	TTAACTACATTGAACGATG
EsopOBP5	ATGGCTGTAAGCGGACTC	GGGCAAGGGAAGGAATTAA
EsopOBP6	CAGTTTATATTAGTTCAGAATGGC	GAAATACTTAATCATTCTCCTT
EsopOBP7	ATGAAGGTCCTCGCCGTCGT	TCAGTCTAATACGGGGAAGG
EsopOBP8	AAGATTAGGTATGTTTTGC	AGCTTATTTTTGTGAAGGC
EsopOBP9	CAAGATCAAAGGTGCAAAG	AGCACGAACTCAAAAATCA
EsopOBP10	AATCCACACTCTCAAAAATG	AAAATTAGTCCAATGGCTGG
EsopOBP11	GACAGTTTATTGCGACGAT	ATTTACACTTGGCAAGCAC
EsopOBP12	GTTTTTGTAACTACGGCTG	GACGAGTGTTGAAGCTATT
EsopOBP13	CCTGCTGATCTTCCTGGGG	ACTTGTGCATGACCTGGGC
EsopOBP14	ATGAAAATTTTTATTGTAGCG	CTTCACACTTGTAAAAGACA
EsopOBP15	CTCTATACAAAAATGGAAACTCA	AAAGGTTATTGGTTGAGCGC
EsopOBP16	CTTTAAAACATATCTTCGT	AGCTTAGAATTTACAACAC

Table S5-4. Primers for *Encarsia sophia* OBP qPCR

OBP	Forward primer (5'-3')	Reverse primer (5'-3')
EsopOBP1	CTATGGGAGTGCTGGACGAT	ACAATTCGCATATTCACACGGA
EsopOBP2	GAGTGCCTCAGAGAGTATGGA	CTTCCTTGCACGCGTTGTAA
EsopOBP3	ACCGGAAGCCCTAGATTTCTT	AGGGTTTATAGTATCGTCAGGGA
EsopOBP4	AAGGACCATCGACACAGGAG	TCAATATAAACGCCATGCCCA
EsopOBP5	GGTCAAGATCTGCCTGACAC	TCAGCTGGTTGGTTTTCAGT
EsopOBP6	TGACGTGCTTCAAAGAATAGGA	TCCAACGTTGTTCAGGCAAA
EsopOBP7	GCTCGACTGCTTCTCTTCCT	TGAATACGTTGCCTCCGGT

EsopOBP8	GAAACCGGAGTAGATTCAGCG	GCTGTTTCACATTTGTCGCC
EsopOBP9	ACGGTTTGACAGAAGCAGATT	CGTTCGCTTTGTTCATGCAC
EsopOBP10	CCCGAAAGTCAAGTGCTTCC	GCACTCGTCGGTCATTTCAT
EsopOBP11	TGGACCTGATGATTGACGGT	TTTACACTTGGCAAGCACGG
EsopOBP12	GAACATGATGGTCGACGGTA	CTTGACGAGTGTTGAAGCTATTT
EsopOBP13	AAACAAGACGCCGAGGATTG	TCTCCATCACGTTGTCCAGG
EsopOBP14	AAAACTTGGACTCATCGCCG	AAAAGACAGCGGCTACTTCG
EsopOBP15	CTCGGCGTATCAATGGACAC	GCTAAATCGACGCACTCCAA
EsopOBP16	AGAGTCTGAACACGCTGGTA	CGTCGCATTTGTCGCTTATT

>EsopOBP1
MKFVGVFCLFLILQINAKEYEESPAVKEIRECYEKYGTVKDEIKLNQKQIT
CNNYCIQKAMGVLDDAGHVDMKGIAEINPSFEREDVERIANICNKDLEGNT
DPCEYANCLQRNNFNFKLGNSPRISKFIHDLKYVIVGYNLLFKFSFDRKPNIF
KKYYFMTMENVF
>EsopOBP2
MKATIVILTFCVAGVFSGIVNNNKSDVNNECLREYGINPDTIYSNPESEESK
LTDEQIYCVAACVYRDHGIMRPNGTIDEEKAESFFGKEDQDERNIFFSVYNA
CKEGNVGCKLAQCMFTELKNHWSSGSSNGSTTDDELEFTSEFTRRLFAGRG
LAQFA
>EsopOBP3
MNLNIIFLLTISFTTSRTWFSPEALDFLYDYELDCMYQSADVTNEDIEELRT
KHIVYDTIKMTKFSLCMLKKFNVVFPDDTINPDVSKYTMPRDYIEVDYATLR
DCKEKGGKDFYEKVRNIMSCFLQRDQLVMAPHSRKGRDTSTNSLTEEDQTT
IKQNF
>EsopOBP4
MREAFDCLFVSAILLAACWSQATSDTRNSTTCVNVSVAINLVDEECVGKS
KTKGPSTQEDENSIDAHNVEEMNAYAVCLLKKSSIMDESGKVNFNFDIVKIV
KNLYKKTDDKGLGMAFILKSVNKCRNTTGSDNSTLATGIIKCLMANQINIVQ
CS-KVKIQLIFLIFNNH-HKEKLYILIKSSYI
>EsopOBP5
MAVSGLLTVCFLALSVTVYSVTAGENGLINECVQELGLTQAQLGSVFGAG
QDLPDTEISNNLFKCNLQKMKVINTDGSLNHDSNTWVDEAWEADKFIKDCF
VKTENQPAEKWGRLIFNCFGKVIIRGPPELFEYAEKQRAKGKGRN
>EsopOBP6
MAFKSFGIFTVCLLALLVNLTRASDPDVECRSKYNIDNDVLQRIGNNPIPD
EENLNNYCTCLFKAWNIINADGSINKDPSTWLGIFTSKVSKEQSKDYPLLVC
LNNVGTEPKEKYARIIGNCFNDVFNNATPEILKKLKGE
>EsopOBP7
MKVLAVVLAICLASCYAATLSDEQKAKLKGYKEACIADSKVDAEVVNAI
LKGGKITREEKLDCFSSCFLKKLGVQKADGTPDANAAIELAKTTNVDVAKA
TEVINKCKDLTGKSDCETGGNVFTCFIENKSFPVLD
>EsopOBP8
MNFKNFKIRYVLLAVASCVVCGAARTFTDKEIAKILSDKEKCIQETGVDSA
VFTLINNYQPFTVTPELKCFWKCILEANNVMKPDGSIDLDDPNDNKEIKACK
KLRDEKGDKCETASTIMICLHKNKLLPILEL
> EsopOBP9
MKFFICALVVLVAATLAYAGLAEDVKKPTSECMTEHGLTEADLKGKIPYQ
DQKIKCFLACIMKKMGMMEDGKFVIEKALEVVSKDKPLDDATQQQSIECM
NKANEQTDECEAAGALYKCQEDAGIIKQPASN
>EsopOBP10

MKAQVCCSALVVLLAVALVSCDQSPEVQKFNDECKSEIGLAEISDNPDIG
DPKVKCFLACMMKKMGKMADGKVVVDKEIEYVMKHIPGADDAMKQKTT
ECITKANEMTDECEVAASMYTCGKENIGQPLD

>EsopOBP11

MKTLFICTLLVFVTRTVYCDDVTPAQIQTATQECTNEWGLTFEEWHNNPT
SDDPKAKCFSACFMKKMDLMIDGKIVKDKGIEMYTKFHQDADDASKQKVI
ACIDKANEQTDECEVASVLAKCKFDAKIN

>EsopOBP12

MKTLLFSAVLVFVTTAVLGGDVSPDLFKTAVQECSKELGMTLEEWDKDS
QSDDPKSKCFMACCMKKLNMMVDGKIEKDRAMELYVHFKPEADDATKLI
YIECINKANVETDECEIASTLVKCKYAAKIE

>EsopOBP13

MKSLLIFLGLLVAVYAGCEIPPEMKQDAEDCAKEVGLADVSRLNQADLVE
QPEKAAVVCMLKKRSMMIDGKLHLDNVMENIMKVYPNLEDIVRPRIVECV
ELANVQFGEEPVAQVMHKCFMEKICSSA

>EsopOBP14

MKIFIVALTIFIGFAALGRAEVKEEDMKNAGYECKHEMKMNQSDFKPKLT
YDNYKMNCYLACYLKKLGLIAEGKLVETEELYFLNSHLELDEDLKRKVAR
CINKANWQSDECEVAAVFYKCE

>EsopOBP15

METHCFIFGALLSFAVIVQCQSESSGVPSIDECASELGVSMDTMNAPDAGT
NPQVKCVLACSLTKEQVMIDGKIKIDEDKVTSDNPEKFLECVDLANQESDEC
ERALYYFKNCIQVPALNQ

>EsopOBP16

MTLKHIFVVLLLAVYAHAGPATDADTIVKECAIELGFPESEHAGIIFNFNAR
CFHKCALEKSAVMINDEFDLDRIFVMIEAKTEPKMKLATACYNQANKISDK
CDAAAHLFRCVVNSKLLI

Chapter 6

General discussion and conclusions

"*Invasive species are the biological wildcards of our age, creating new ecosystems, new combinations of species, and new ecological challenges*."

Richard Hobbs

1. General discussion

The reproductive fitness of parasitoid wasps refers to their ability to successfully complete their life cycle and effectively transmit their genes to the next generation through parasitic behavior under specific environmental conditions. This reproductive fitness reflects the adaptability and competitiveness of parasitoid wasps within an ecosystem and serves as a crucial indicator for evaluating parasitoid population dynamics and their potential in biological control (Quicke, 1997; Lucie et al., 2021; Alena et al., 2022). The evolution of life-history traits has led to complex adaptive strategies aimed at maximizing fitness within local environments (Stearns, 1992; Ye et al., 2024). In natural conditions, parasitoids face limited resources for their offspring's development, along with challenges such as intraspecific and interspecific competition, variations in host quality and density, food scarcity, and host defenses. In response to these challenges, each parasitoid employs specific reproductive strategies to maximize its fitness (Grillenberger, 2009).

Heteronomous hyperparasitoids are a unique group of parasitoid wasps, predominantly found in the genera *Encarsia* of the Aphelinidae family, as well as *Coccophagus*, *Coccophagoides*, and *Coccobius*. These wasps act as natural enemies to pests such as scale insects, whiteflies, and aphids (Hunter and Woolley, 2001; Williams, 1996a). The developmental mode of heteronomous hyperparasitoids resembles that of other primary haplodiploid parasitoids, where fertilized diploid eggs develop into females and unfertilized haploid eggs develop into males. However, what distinguishes them is their heteronomous development, with males and females requiring different host insects for their development. Females, which are primary parasitoids, develop from fertilized eggs and parasitize primary host target pests, obtaining nutrients by feeding on the nymphs or larvae of the pest to complete their development. In contrast, males develop from unfertilized eggs as hyperparasitoids, parasitizing secondary hosts—those previously parasitized by primary parasitoids—and feed on the primary parasitoid larvae to complete their own development (Walter, 1983; Mills and Gutierrez, 1996; Hunter and Woolley, 2001).

The complex reproductive strategy of heteronomous hyperparasitoids presents significant challenges, particularly at the beginning of the growing season or in new habitats where the lack of secondary hosts may result in highly female-biased populations, or even the inability to produce offspring. This raises a critical evolutionary question: how, over the course of long-term evolution and natural selection, have these parasitoids adapted to different hosts and avoided being selected out? To address this, we studied the host adaptability of *E. sophia*, a dominant parasitoid of *B. tabaci*, as a representative species of heteronomous hyperparasitoids. To explore their ability to adjust sex ratios in complex environments, we designed Chapter 3: *Can heteronomous hyperparasitoids recognize host abundance and adjust offspring ratio?* In response to the limitations in understanding the mechanisms behind host adaptation in this group, we developed Chapter 4: *A chromosome-level genome assembly of the heteronomous hyperparasitoid wasp Encarsia sophia*. Finally, building on the findings from Chapters 3 and 4, we formulated Chapter 5: *Daughter*

or Son? Host Odor Determines Offspring Sex in Parasitoid, to investigate the role of host odors in sex determination.

1.1 Sex allocation under different host resources

Parasitoid wasps typically adopt a haplodiploid sex determination system, where unfertilized eggs produce males, and fertilized eggs result in females. Consequently, females can precisely regulate the sex ratio of their offspring by controlling fertilization (West, 2009; Wajnberg, 2012). This adjustment in sex ratio is generally driven by the aim of maximizing reproductive fitness, which is measured by the increase in the number of offspring in the next generation. The sex ratio, in this context, reflects the ratio of males to females in the first generation (Hamilton, 1967). According to the theory of local mate competition (LMC), parasitoids reduce the proportion of male offspring under limited resources to minimize competition among brothers, thereby increasing the proportion of female offspring. This strategy is particularly evident in host patches where multiple females oviposit. The theoretical model predicts that when "n" females oviposit in the same host patch, the optimal male ratio should be "(n-1)/2n" (Hamilton, 1967). This theory applies to the life history patterns of most parasitoid species, where the mother adjusts the number of male offspring to reduce male competition and prioritize female production (Sean et al., 2002; West et al., 2003). Fisher's foundational theory on sex ratio regulation suggests that under conditions of large population sizes and random mating between both sexes, the offspring sex ratio should correspond to the proportion of resources allocated to male and female offspring, with equal numbers of both sexes being a stable evolutionary strategy. Therefore, parents should invest equally in male and female offspring, resulting in a sex ratio of 1:1 (Fisher, 1930).

Do heteronomous parasitoids, a unique type of parasitoid, possess the ability to adjust their offspring sex ratio? And do traditional sex ratio theories apply to them? Our results indicate that *E. sophia* does indeed exhibit sex ratio adjustment, with the offspring sex ratio being significantly influenced by host resource conditions. When the proportion of secondary hosts exceeds that of primary hosts or when host density is low (host limitation), *E. sophia* adjusts the sex ratio of its offspring based on the relative abundance of primary and secondary hosts. However, when the proportion of secondary hosts is low (<0.5) and host density is high, the offspring sex ratio tends toward 1:1. This finding diverges from previous reports on heteronomous parasitoids. Godfray and colleagues suggested that the sex ratio of heteronomous parasitoids trends toward 1:1 as host density increases, regardless of the relative abundance of primary and secondary hosts. In contrast, Walter and Donaldson proposed that the sex ratio is determined solely by the relative abundance of primary and secondary hosts (Godfray and Hunter, 1992, 1994; Walter and Donaldson, 1994). These earlier studies focused on either host density or host proportion as singular factors without considering a more comprehensive view. In nature, however, parasitoids are typically influenced by multiple host factors simultaneously.

Further analysis revealed that as host density increases, *E. sophia's* host-feeding behavior undergoes significant changes. In particular, under conditions of abundant

host resources, female parasitoids increase feeding on primary hosts, leading to a reduction in female offspring and a rise in the proportion of male offspring, causing the sex ratio of the offspring to approach 1:1. This indicates that female behavioral choices under different host densities directly impact offspring sex allocation. Additionally, the relative proportion of secondary hosts significantly influences parasitism behavior. When the proportion of secondary hosts is too high, females tend to reduce parasitism, likely to avoid interspecific competition. This phenomenon suggests that changes in host type and availability not only affect female host selection but also play a crucial role in determining the sex ratio of their offspring. Moreover, the relationship between host encounter rates and offspring sex ratio reflects a complex behavioral mechanism. Hunter (1993) proposed that the sex ratio of *E. pergandiella* might be linked to the proportion of females encountering secondary hosts, while Avilla (1987) suggested that differences in parasitism between primary and secondary hosts could be attributed to variations in the encounter rate and handling time of heteronomous parasitoids toward the two host types (Avilla and Copland, 1987; Hunter, 1993). Our results show that while *E. sophia* has a higher encounter rate with secondary hosts, this does not directly correspond to its offspring sex ratio but instead reflects a preference for secondary hosts. This suggests that after encountering secondary hosts, female parasitoids do not always choose to parasitize but make complex behavioral decisions based on host conditions, which further influence the distribution of offspring sex ratios.

Our study not only enriched the theoretical understanding of sex allocation regulation mechanisms in heteronomous hyperparasitoids but also provides important insights for their large-scale application in biological control. Specifically, in mass rearing, the proper adjustment of host density and the proportion of secondary hosts can effectively increase the production of female offspring, thereby maximizing the efficiency of biological control. For example, when the secondary host proportion is set at 0.2 and the host density at 3/ cm^2, *E. sophia* is able to maximize the production of female offspring with minimal consumption of secondary hosts. This flexible sex ratio adjustment mechanism not only provides optimal conditions for the large-scale production of heteronomous hyperparasitoids but also significantly enhances *E. sophia*'s pest control effectiveness in the field, particularly in unpredictable host environments, thereby giving it strong biological control potential across diverse ecosystems.

1.2 Chromosome-level genome sequencing and assembly

Using Illumina, PacBio HiFi, and Hi-C sequencing technologies, we successfully assembled the chromosome-level genome of *E. sophia*. The genome size is 398.3 Mb, organized into five chromosomes, with a mounting rate of 95.13%. Repetitive sequences constitute 54.59% of the genome, and a total of 14,914 protein-coding genes were predicted, with 95.5% of the genes functionally annotated. This is the first genome obtained for a heteronomous hyperparasitoid wasp.

The significant expansion and contraction of gene families are often associated with the adaptive evolution of species (Wu, Zhang et al., 2019; Zhang et al., 2020). In the

genome of *A. gifuensis*, 405 homologous groups have been notably expanded and 663 have contracted in comparison to the most recent common ancestor (MRCA) of *A. gifuensis* and *F. arisanus* (Li et al., 2020). In the annotated gene models of *Chelonus formosanus*, 355 gene families were identified as expanded (with 58 significantly expanded), while 383 were contracted (with 28 significantly contracted) (Liu et al., 2022). In *Theocolax elegans*, 130 gene families experienced significant expansion events, and 34 gene families underwent significant contraction events (Xiao et al., 2023). Comparative genomic analysis of *E. sophia* revealed 1,000 significantly expanded homologous groups and 1,918 significantly contracted homologous groups when compared to its MRCA, along with the identification of 105 positively selected genes. These genes are primarily involved in immune defense pathways, metabolic processes, and chemosensory systems. The number of significantly expanded and contracted genes in *E. sophia* is notably higher than that in other primary parasitoids, suggesting that these genes may play a crucial role in the evolution of *E. sophia*'s heteronomous hyperparasitism traits.

Odorant receptor (OR) genes provide important insights into the host recognition mechanisms in insects (Wang et al., 2020). A total of 56 OR genes were annotated and mapped to the chromosomes. These genes may be associated with the host selection process in *E. sophia*, particularly in its heteronomous hyperparasitism. Some of the ORs in *E. sophia* clustered phylogenetically with those in *Eretmocerus hayati*, such as *EsopOR18*, *EsopOR22*, and *EsopOR39*, as both species parasitize *B. tabaci*, suggesting that these genes may be involved in the recognition of this host (Zhong et al., 2023). Additionally, ORs specific to *E. sophia*, such as *EsopOR24*, *EsopOR26*, *EsopOR40*, and *EsopOR41*, may play a role in the recognition of secondary hosts. This work provides essential genomic data for understanding the unique evolutionary traits and environmental adaptability of heteronomous hyperparasitoid wasps, offering crucial insights into how parasitoid wasps achieve precise host recognition and sex ratio regulation through molecular mechanisms. This genome-level research not only lays a foundation for optimizing biological control applications using molecular tools but also opens up broad possibilities for exploring parasitoid behavioral decision-making through genomics.

1.3 Behavioral decision-making mechanism of heteronomous oviposition

In the behavioral studies of parasitoid wasps, host selection and the regulation of offspring sex ratios have consistently been key research areas. Parasitoid wasps rely on complex sensory mechanisms to recognize hosts, which is crucial for ensuring the survival of their offspring (Godfray, 1993; Kafle et al., 2020). Different types of stimuli, such as chemical, visual, and tactile cues, are used by parasitoids to identify their hosts (Jiang et al., 2024). Typically, hosts that have already been parasitized are considered low-quality resources due to limitations in nutrients and space, and most parasitoids avoid laying eggs on parasitized hosts. This recognition behavior reduces intraspecific competition and increases the survival chances of their offspring (van Alphen & Visser, 1990; Ruschioni et al., 2015). As a heteronomous parasitoid, *E.*

sophia exhibits clear host selection behavior by laying female eggs in primary hosts (*B. tabaci*) and male eggs in secondary hosts (previously parasitized primary hosts). Thus, it relies on sensory signals to distinguish between host types and make oviposition decisions.

Behavioral studies on parasitoid wasps' host selection have shown that females initially probe the host surface with their antennae, followed by inserting their ovipositor into the host to further assess whether to lay eggs (Tamò et al., 2006; Kafle et al., 2020). This behavior is also observed in *E. sophia*. We found that the ovipositor probing time on secondary hosts was significantly longer than on primary hosts, suggesting that *E. sophia* relies on ovipositor insertion to distinguish between different host types. Further observation using scanning electron microscopy revealed that the ovipositor of female *E. sophia* is equipped with well-developed physical and chemical receptors, including Trichoid Sensilla and Böhm's bristles. This indicates that host discrimination is not only based on physical contact but also on the perception of chemical signals through these receptors.

It has traditionally been believed that *E. sophia* can only lay male eggs in dry secondary host environments, suggesting that the parasitoid's sex allocation might be influenced by the physical pressure within the host (Gerling, 1983; Hunter and Woolley, 2001). However, our findings indicate that *E. sophia* can make sex allocation decisions based on host type, regardless of the host's internal fluid conditions. This overturns previous assumptions and suggests that physical factors are not the primary determinant of sex allocation (Fatouros et al., 2005; Khatri et al., 2021). We found that *E. sophia* females detect volatile compounds from hosts through olfactory receptors on their ovipositors. By analyzing the volatiles of four secondary hosts and one primary host, we identified n-heptacosane, a compound unique to secondary hosts, which induces the laying of male eggs. Additionally, we identified two key odorant-binding proteins, *EsopOBP1* and *EsopOBP10*, located on the ovipositor. These proteins have a high affinity for n-heptacosane and assist the female wasps in recognizing different host types.

This is the first time functional protein genes have been identified on the ovipositor of Hymenopteran parasitoids, marking a significant advancement in understanding the molecular mechanisms behind parasitoid sex allocation. This discovery not only opens new avenues for regulating sex allocation through olfactory compounds but also provides new insights into how parasitoid wasps make behavioral decisions in complex ecosystems. The potential applications of chemical sensing in biological control are highlighted, and future research could explore leveraging these olfactory signals to manipulate oviposition behavior, enabling precise sex allocation control and offering technical support for large-scale pest management strategies.

2. Conclusions and perspectives

As the dominant parasitoid of the whitefly *B. tabaci*, *E. sophia* has long faced reproductive bottlenecks due to its unique heteronomous parasitism reproductive strategy. This study systematically investigated the reproductive adaptive strategies of *E. sophia* using a combination of behavioral, physiological, genomic,

transcriptomic, and molecular approaches. The research revealed the molecular mechanisms underlying sex ratio regulation, genomic characteristics, and oviposition decision-making. *E. sophia* can adjust the sex ratio of its offspring based on host resource conditions, such as host density and the proportion of secondary hosts, to optimize its reproductive fitness. Under conditions where secondary hosts outnumber primary hosts or when host density is low (host limitation), the offspring sex ratio is adjusted according to the relative abundance of primary and secondary hosts. However, when the proportion of secondary hosts is low (<0.5) and host density is high, the offspring sex ratio approaches 1:1. This regulatory mechanism improves biological control efficiency under low host density conditions and provides practical reference for large-scale application and field release. For instance, when the secondary host proportion is 0.2 and host density is 30/9.6 cm^2, *E. sophia* maximizes female offspring production while minimizing the consumption of secondary hosts. A chromosome-level genome assembly of *E. sophia* was completed, revealing a genome size of 398.3 Mb, assembled into five chromosomes with a mapping rate of 95.13%, and predicting 14,914 protein-coding genes. This represents the first complete genome for heteronomous hyperparasitoids, offering critical genomic information for understanding its adaptive evolutionary mechanisms and host interactions. Comparative genomics identified 1,000 significantly expanded homologous groups, 1,918 significantly contracted homologous groups, and 105 positively selected genes. Additionally, 56 odorant receptor (OR) genes were identified, providing valuable genomic insights for further research into host interactions and adaptive evolution. The study also found that *E. sophia* females rely on olfactory receptors on their ovipositors to detect the secondary host-specific volatile n-heptacosane, which induces the laying of male eggs. Two key odorant-binding proteins, *EsopOBP1* and *EsopOBP10*, play a crucial role in this process. This mechanism of oviposition decision-making, regulated by chemical signals, offers the potential for optimizing the application of parasitoids in biological control strategies.

Based on the findings of this study, future research can focus on the following aspects:

1) Optimization of mass rearing techniques: The study provides a theoretical foundation for the large-scale propagation of *E. sophia* by identifying optimal rearing conditions. By optimizing host resource allocation and environmental factors, large-scale production of *E. sophia* could be achieved. In particular, when the secondary host proportion is set at 0.2 and the host density at 3/ cm^2, the production of female offspring can be maximized while minimizing the consumption of secondary hosts.

2) Control of *E. sophia* offspring sex ratio through host volatiles: Chemical signals play a crucial role in the host selection and oviposition decisions of *E. sophia*. Specifically, n-heptacosane significantly influences its behavior in producing male offspring. Future research could explore how manipulating host volatiles might regulate the sex ratio of parasitoids, providing new methods for precisely controlling parasitoid behavior in pest management. Moreover, it would be worthwhile to investigate whether the influence of such chemical signals on sex determination extends beyond this specific group of parasitic insects.

3) In-depth functional gene analysis: The transcriptomic and genomic data uncovered in this study lay the groundwork for future research into gene functions. Further exploration of the genes involved in sex allocation and host selection in *E. sophia*, particularly olfactory receptors and odorant-binding proteins, could support applications in gene editing and other techniques to enhance reproductive efficiency and the biological control potential of parasitoids.

4) Expanding the application of heteronomous hyperparasitoids in biological control: As understanding of the reproductive adaptive strategies and molecular mechanisms of *E. sophia* deepens, its potential in biological control will be further enhanced. Future research could integrate ecological, molecular, and genomic approaches to develop more efficient and sustainable pest management strategies.

References

"*Once an invasive species gains a foothold, it can spread like wildfire, disrupting ecosystems and outcompeting native species.*"

David Suzuki

Aalto AL, Luukkonen V, Meinander A. Ubiquitin signalling in Drosophila innate immune responses. FEBS J. 2023 Dec 8. doi: 10.1111/febs.17028. Epub ahead of print. PMID: 38069549.

Abe J, Iritani R, Tsuchida K, Kamimura Y, West SA. (2021). A solution to a sex ratio puzzle in *Melittobia* wasps. *Proc Natl Acad Sci U S A*, *118*(20), e2024656118. https://doi.org/10.1073/pnas.2024656118

Ahmed, T., Zhang, T.T., Wang, Z.Y., He, K.L., and Bai, S.X. (2013). Morphology and ultrastructure of antennal sensilla of *Macrocentrus cingulum* Brischke (Hymenoptera: Braconidae) and their probable functions. Micron. 50, 35-43.

Alena Samková, Jan Raška, Jiří Hadrava, Jiří Skuhrovec. An intergenerational approach to parasitoid fitness determined using clutch size. Scientific Reports. 2022; 12(1): 0-0. https://doi.org/10.1038/s41598-022-09024-z.

Allen GR, Kazmer DJ, Luck RF. Postcopulatory male-behaviour, sperm precedence and multiple mating in a solitary parasitoid wasp. Animal Behaviour. 1994; 48: 635–644.

Andersson M, Simmons LW. Sexual selection and mate choice. Trends in Ecology & Evolution. 2006; 21(6): 296-302. DOI: 10.1016/j.tree.2006.03.015.

Ankersmit, G., Agreman, T., and Dijkman, H. (1981). Parasitism of colour forms in *Sitobion avenae*. Entomol. Exp. Appl. 29, 362-363.

Ankersmit, G., Bell, C., Dijkman, H., Mace, N., Rietstra, S., Schroeder, J., and Devisser, C. (1986). Incidence of parasitism by *Aphidius rhopalosiphi* in color forms of the aphid *Sitobion avenae*. Entomol. Exp. Appl. 40, 223-229.

Antolin MF, Ode PJ, Heimpel GE, O'Hara RB, Strand MR. Population structure, mating system, and sex-determining allele diversity of the parasitoid wasp *Habrobracon hebetor*. Heredity (Edinb). 2003; 91(4): 373-81. doi: 10.1038/sj.hdy.6800337.

Arnqvist G, Nilsson T. The evolution of polyandry: multiple mating and female fitness in insects. Animal Behaviour. 2000; 60(2): 145-164. DOI: 10.1006/anbe.2000.1446.

Arthur, A.P. (1981). Host acceptance by parasitoids, pp. 97–120, in D.A. Nordlund, R.L. Jones, and W.J. Lewis (eds.). Semiochemicals. Their Role in Pest Control. Wiley, New York.

Aruna AS, Manjunath D. Reproductive performance of *Nesolynx thymus* (Hymenoptera: Eulophidae) in relation to age of *Musca domestica* (Diptera: Muscidae). Biocontrol Sci Tech. 2009; 19(2): 139–149.

Asgari S, Rivers DB. Venom proteins from endoparasitoid wasps and their role in host-parasite interactions. Annu Rev Entomol. 2011; 56: 313-35. doi: 10.1146/annurev-ento-120709-144849.

Asgari S, Zhang G, Zareie R, Schmidt O. A serine proteinase homolog venom protein from an endoparasitoid wasp inhibits melanization of the host hemolymph.

Insect Biochem Mol Biol. 2003; 33(10): 1017-24. doi: 10.1016/s0965-1748(03)00116-4.

Askari Seyahooei M, Kraaijeveld K, Bagheri A, van Alphen JJM. Adult size and timing of reproduction in five species of *Asobara* parasitoid wasps. Insect Sci. 2020; 27(6): 1334-1345. doi: 10.1111/1744-7917.12728.

Askew RR, Shaw MR. Parasitoid communities: their size, structure and development. Insect Parasitoids. 1986.

Aspin E, Keller MA, Hardy ICW. The 'Goldilocks Grub': reproductive responses to leafroller host development in *Goniozus jacintae*, a parasitoid of the light brown apple moth. Bull Entomol Res. 2024; 1-9. doi: 10.1017/S0007485324000348. Epub ahead of print. PMID: 39263761.

Avilla J, Copland MJW. (1987). Effects of host stage on the development of the facultative autoparasitoid *Encarsia tricolor* (Hymenoptera: Aphelinidae). *Annals of Applied Biology*, *110*(2), 381-389. https://doi.org/10.1111/j.1744-7348.1987.tb03269.x

Ayala A, Toledo J, Pérez-Lachaud G, Liedo P, Montoya P. Superparasitism and fitness parameters in three native wasp parasitoids (Braconidae: Opiinae) of the Mexican fruit fly, *Anastrepha ludens* (Diptera: Tephritidae). Bull Entomol Res. 2022; 112(2): 253-260. doi: 10.1017/S000748532100081X.

Ayelo, P.M., Yusuf, A.A., Chailleux, A., Mohamed, S.A., Pirk, C.W.W., and Deletre, E. (2022). Chemical Cues From Honeydew and Cuticular Extracts of *Trialeurodes vaporariorum* Serve as Kairomones for The Parasitoid *Encarsia formosa*. J Chem Ecol. 48, 370-383.

Baghery F, Michaud JP, Dini A. Tritrophic effects mediate host suitability for two parasitoids of the carob moth (Lepidoptera: Pyralidae), infesting pistachio kernels. Environ Entomol. 2024; 53(1): 94-100. doi: 10.1093/ee/nvae004.

Bairoch A, Apweiler R. The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. Nucleic Acids Res. 2000;28(1):45-8. https://doi.org/10.1093/nar/28.1.45

Bao W, Kojima KK, Kohany O. Repbase Update, a database of repetitive elements in eukaryotic genomes. Mob DNA. 2015;6:11. https://doi.org/10.1186/s13100-015-0041-9

Barratt BI, Murney R, Easingwood R, Ward VK. Virus-like particles in the ovaries of *Microctonus aethiopoides* Loan (Hymenoptera: Braconidae): comparison of biotypes from Morocco and Europe. J Invertebr Pathol. 2006; 91(1): 13-8. doi: 10.1016/j.jip.2005.10.008.

Battaglia, D., Pennacchio, F., Marincola, G., and Tranfaglia, A. (1993) Cornicle secretion of *Acyrthosiphon pisum* (Homoptera: Aphididae) as a contact kairomone for the parasitoid *Aphidius ervi* (Hymenoptera Braconidae). Eur. J. Entomol. 90, 423-428.

Battaglia, D., Poppy, G., Powell, W., Romano, A., Tranfaglia, A., and Pennacchio, F. (2000). Physical and chemical cues influencing the oviposition behaviour of *Aphidius ervi*. Entomol. Exp. Appl. 94, 219-227.

Beckage NE, Gelman DB. Wasp parasitoid disruption of host development: implications for new biologically based strategies for insect control. Annu Rev Entomol. 2004; 49(1): 299-301.

Belaghzal H, Dekker J, Gibcus JH. Hi-C 2.0: An optimized Hi-C procedure for high-resolution genome-wide mapping of chromosome conformation. Methods, 2017;123:56-65. https://doi.org/10.1016/j.ymeth.2017.04.004

Bell HA, Weaver RJ. Ability to host regulate determines host choice and reproductive success in the gregarious ectoparasitoid *Eulophus pennicornis* (Hymenoptera: Eulophidae). Phys Ent. 2008; 33: 62–67.

Benson G. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res. 1999;27(2):573-80. https://doi.org/10.1093/nar/27.2.573

Bernstein C, Jervis MA. Food-searching in parasitoids: the dilemma of choosing between 'immediate' or future fitness gains. In: Behavioral Ecology of Insect Parasitoids. Wajnberg E, van Bernstein C, J. Alphen (Eds.), Blackwell Publishing, Malden, MA. 2006; pp. 129-171.

Betty Benrey. The effects of plant domestication on the foraging and performance of parasitoids. Curr Opin Insect Sci. 2023; 57(0): 101031. doi: 10.1016/j.cois.2023.101031.

Bezemer TM, Mills NJ. Clutch size decisions of a gregarious parasitoid under laboratory and field conditions. Animal Behaviour. 2003; 66: 1119-1128.

Bichang'a, G., Da Lage, J.L, Capdevielle-Dulac, C., Zivy, M., Balliau, T., Sambai, K., Le Ru, B., Kaiser, L., Juma, G., Maina, ENM., and Calatayud, P.A. (2018) α-Amylase Mediates Host Acceptance in the Braconid Parasitoid *Cotesia flavipes*. J Chem Ecol. 44, 1030-1039.

Bin, F., and Vinson, S.B. (1986). Morphology of the antennal sex-gland in male *Trissolcus basalis* (Woll.) (Hymenoptera: Scelionidae), an egg parasitoid of the green stink bug, *Nezara viridula* (Hemiptera: Pentatomidae). Int J Morphol. 15, 129-138.

Birney E, Clamp M, Durbin R. GeneWise and Genomewise. Genome Res. 2004;14(5):988-95. https://doi.org/10.1101/gr.1865504

Bleeker, M.A, Smid, H.M, Van Aelst, A.C., Van Loon, J.J., and Vet, L.E. (2004). Antennal sensilla of two parasitoid wasps: a comparative scanning electron microscopy study. Microsc Res Tech. 63, 266-273.

Böckmann EA, Tormos J, Beitia F, Fischer K. Offspring production and self-superparasitism in the solitary ectoparasitoid Spalangia cameroni (Hymenoptera: Pteromalidae) in relation to host abundance. Bull Entomol Res. 2012; 102: 131–137. https://doi.org/10.1017/S0007485311000447.

Boivin G, Fauvergue X, Wajnberg E. Optimal patch residence time in egg parasitoids: innate versus learned estimate of patch quality. Oecologia. 2004; 138(4): 640-647. doi: 10.1007/s00442-003-1469-z.

Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114-20. https://doi.org/10.1093/bioinformatics/btu170.

Bollinger EK, Harper SJ, Micheal KJ, Barrett GW. Avoidance of inbreeding in the meadow vole (*Microtus pennsylvanicus*). J Mammal. 1991; 72(2): 419-421. doi: 10.2307/1382117.

Bon VJ, Moral RD, Reigada C. (2022). Influence of intra- and inter-specific competition between egg parasitoids on the effectiveness of biological control of *Euschistus heros* (Hemiptera: Pentatomidae). *Biologal control*, 170, 104903. https://doi.org/10.1016/j.biocontrol.2022.104903

Boomsma J. Split sex ratios and queen-male conflict over sperm allocation. Proc Biol Sci. 1996; 263(1371): 697-704.

Boulton RA, Collins LA, Shuker DM. Beyond sex allocation: the role of mating systems in sexual selection in parasitoid wasps. Biol Rev Camb Philos Soc. 2015; 90(2): 599-627. doi: 10.1111/brv.12126.

Boulton RA, Shuker DM. A sex allocation cost to polyandry in a parasitoid wasp. Biol Lett. 2015; 11(6): 20150205. doi: 10.1098/rsbl.2015.0205.

Boulton RA, Shuker DM. The costs and benefits of multiple mating in a mostly monandrous wasp. Evolution. 2015; 69(4): 939-949. doi: 10.1111/evo.12636.

Brantley SE, Stouthamer CM, Kr P, Fischer ML, Hill J, Schlenke TA, Mortimer NT. Host JAK-STAT activity is a target of parasitoid wasp virulence strategies. PLoS Pathog. 2024; 20(7): e1012349. doi: 10.1371/journal.ppat.1012349.

Bressac C, Khanh HDT, Chevrier C. Effects of age and repeated mating on male sperm supply and paternity in a parasitoid wasp. Entomol Exp Appl. 2009; 130: 207–213.

Bretman A, Gage MJG, Chapman T. Quick-change artists: male plastic behavioural responses to rivals. Trends Ecol Evol. 2011; 26(9): 467-473. doi: 10.1016/j.tree.2011.05.002.

Briggs CJ, Collier TR. Autoparasitism, interference, and parasitoid-pest population dynamics. Theor Popul Biol. 2001; 60: 33-57.

Buitenhuis, R., Mcneil, J.N., Boivin, G., and Brodeur, J. (2004). The Role of honeydew in Host Searching of Aphid Hyperparasitoids. J. Chem. Ecol. 30, 273-285.

Burke GR, Sharanowski BJ. Parasitoid wasps. Curr Biol. 2024; 34: 473-493.

Busiere LF, Gwynne DT, Brooks R. Contrasting sexual selection on males and females in a role-reversed swarming dance fly, *Rhamphomyia longicauda*

Loew (Diptera: Empididae). J Evol Biol. 2008; 21: 1683-1691. doi: 10.1111/j.1420-9101.2008.01580.x.

Butcher RDJ, Whitfield WGF, Hubbard SF. Complementary sex determination in the genus *Diadegma* (Hymenoptera: Ichneumonidae). J Evol Biol. 2000; 13: 593-606. doi: 10.1046/j.1420-9101.2000.00203.x.

Cabello T, Gallego JR, Lopez I, Gamez M, Garay J. Importance of Host Feeding in the Biological Control of Insect Pests: Case Study of Egg Parasitoid Species (Hymenoptera: Chalcidoidea: Trichogrammatidae). Insects. 2024; 15(7): 496. doi: 10.3390/insects15070496.

Camacho C, Coulouris G, Avagyan V, et al. BLAST+: architecture and applications. BMC Bioinformatics 2009,15(10):421. https://doi.org/10.1186/1471-2105-10-421

Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics. 2009;25(15):1972-3. https://doi.org/10.1093/bioinformatics/btp348

Carneiro TR, Fernandes AO, Cruz I. Influência da competição intra-específica entre fêmeas e da ausência de hospedeiro no parasitismo de *Telenomus remus* Nixon (Hymenoptera, Scelionidae) sobre ovos de *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera, Noctuidae). Rev Bras Entomol. 2009; 53: 482–486.

Caspary R, Wosula EN, Issa KA, et al. Cutting Dipping Application of Flupyradifurone against Cassava Whiteflies *Bemisia tabaci* and Impact on Its Parasitism in Cassava. Insects. 2023;14(10):796. https://doi.org/10.3390/insects14100796

Cate RH, Sauer JR, Eikenbary RD. Demonstration of host feeding by the parasitoid *Aphelinus asychis* (Hymenoptera: Eulophidae). Entomophaga. 1974; 19: 479-482.

Cerqueira de Araujo A, Leobold M, Bézier A, Musset K, Uzbekov R, Volkoff AN, Drezen JM, Huguet E, Josse T. Conserved viral transcription plays a key role in virus-like particle production of the parasitoid wasp *Venturia canescens*. J Virol. 2022; 96(13): e0052422. doi: 10.1128/jvi.00524-22.

Chamov EL, Losden Hartogh RL, Jones WT, Assem JVD. Sex ratio evolution in a variable environment. Nature. 1981; 289: 27-33.

Chan MS, Godfray HCJ. Host-feeding strategies of parasitoid wasps. Evol Ecol. 1993; 7: 593-604.

Chan PP, Lin BY, Mak AJ, et al. tRNAscan-SE 2.0: improved detection and functional classification of transfer RNA genes. Nucleic Acids Res. 2021;49(16):9077-9096. https://doi.org/10.1093/nar/gkab688

Chapman, R.F. (1998). *The Insects: Structure and Function*, 4th edn. Cambridge University Press, Cambridge, UK.

Charles O. A review of management of major arthropod pests affecting cassava production in Sub-Saharan Africa. Crop Prot. 2024;175:1-15. https://doi.org/10.1016/j.cropro.2023.106465.

Charnov EL, Skinner SW. Evolution of host selection and clutch size in parasitoid wasps. Fla Entomol. 1984; 67: 5-21.

Charnov EL. Optimal foraging: the marginal value theorem. Theor Popul Biol. 1976; 9: 129-136.

Chavarín-Gómez LE, Torres-Enciso P, Palmeros-Suárez PA, Ramirez-Romero R. Influence of the number of hosts and the risk of predation on the foraging behavior of the parasitoid *Eretmocerus eremicus*. Pest Manag Sci. 2023; 79(11): 4208-4218. doi: 10.1002/ps.7617.

Chen C, Wu Y, Li J, et al. TBtools-II: A "one for all, all for one" bioinformatics platform for biological big-data mining. Mol Plant. 2023;16(11):1733-1742. https://doi.org/10.1016/j.molp.2023.09.010

Chen C, Zhao H, Zhou P, Wang Q. Parasitoid-host interaction behaviors in relation to host stages in the Tamarixia triozae (Hymenoptera: Eulophidae)–Bactericera cockerelli (Hemiptera: Triozidae) system. J Insect Sci. 2024; 24(1): 0-0. https://doi.org/10.1093/jisesa/ieae016.

Chen Y, Liu XG, Wang J, Zhao J, Lu ZX, Liu YH. *Cotesia ruficrus* (Hymenoptera: Braconidae) parasitizing *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae): developmental interactions and food utilization efficiency of hosts. J Econ Entomol. 2016; 109(2): 588-593. doi: 10.1093/jee/tov405.

Cheng H, Concepcion GT, Feng X, et al. Haplotype-resolved de novo assembly using phased assembly graphs with hifiasm. Nat Methods. 2021;18(2):170-175. https://doi.org/10.1038/s41592-020-01056-5

Chevrier C, Bressac C. Sperm storage and use after multiple mating in *Dinarmus basalis* (Hymenoptera: Pteromalidae). J Insect Behav. 2002; 15(3): 385-398. doi: 10.1023/A:1016269210140.

Cinege G, Fodor K, Magyar LB, Lipinszki Z, Hultmark D, Andó I. Cellular immunity of *Drosophila willistoni* reveals novel complexity in insect anti-parasitoid defense. Cells. 2024; 13(7): 593. doi: 10.3390/cells13070593.

Cloutier C, Duperron J, Tertuliano M, Mcneil JN. Host instar, body size, and fitness in the koinobiotic parasitoid *Aphidius nigripes*. Entomol Exp Appl. 2010; 97(1).

Cock PJ, Fields CJ, Goto N, et al. The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic Acids Res. 2010;38(6):1767-71. https://doi.org/10.1093/nar/gkp1137

Colgan P, Taylor P. (1981). Sex-ratio in autoparasitic Hymenoptera. *The American Naturalist*, *117*(4), 564-566. https://www.journals.uchicago.edu/doi/abs/10.1086/283738?journalCode=an

Consoli FL, Lewis D, Keeley L, Vinson SB. Characterization of a cDNA encoding a putative chitinase from teratocytes of the endoparasitoid *Toxoneuron nigriceps*. Entomol Exp Appl. 2007; 122: 271-278. doi: 10.1111/j.1570-7458.2006.00514.x.

Cowan DP, Stahlhut JK. Functionally reproductive diploid and haploid males in an inbreeding hymenopteran with complementary sex determination. Proc Natl Acad Sci U S A. 2004; 101(28): 10374-10379. doi: 10.1073/pnas.0303976101.

Crozatier M, Ubeda JM, Vincent A. Cellular immune response to parasitization in *Drosophila* requires the EBF orthologue Collier. PLoS Biol. 2004; 2(8): e196.

Daane KM, Wang X, Duerr SS, Kuhn EJ, Son Y, Yokota GY. Biology of *Habrobracon gelechiae* (Hymenoptera: Braconidae), as a parasitoid of the obliquebanded leafroller (Lepidoptera: Tortricidae). Environ Entomol. 2013; 42(1): 107-115. doi: 10.1603/EN12166.

Dahms, E.C. (1984). An interpretation of the structure and function of the antennal sense organs of *Melittobia australica* (Hymenoptera: Eulophidae) with the discovery of a large dermal gland in the male scape. Memoirs of the Queensland Museum. 21, 361-385.

Damien M, Barascou L, Ridel A, Van Baaren J, Le Lann C. Food or host: do physiological state and flower type affect foraging decisions of parasitoids? Behav Ecol Sociobiol. 2019; 73(11).

Damiens D, Boivin G. Male reproductive strategy in *Trichogramma evanescens*: sperm production and allocation to females. Physiol Entomol. 2005; 30: 241-247.

Danielsson I. Antagonistic pre-copulatory and post-copulatory sexual selection on male body size in a water strider (*Gerris lacustris*). Proc Biol Sci. 2001; 268: 77-81.

De Bie T, Cristianini N, Demuth JP, et al. CAFE: a computational tool for the study of gene family evolution. Bioinformatics. 2006;22(10):1269-71. https://doi.org/10.1093/bioinformatics/btl097

de Graaf DC, Aerts M, Brunain M, Desjardins CA, Jacobs FJ, Werren JH, et al. Insights into the venom composition of the ectoparasitoid wasp *Nasonia vitripennis* from bioinformatic and proteomic studies. Insect Mol Biol. 2010; 19(Suppl 1): 11-26.

De Moraes, C.M., Lewis, W.J., Paré, P.W., Alborn, H.T., and Tumlinson, J.H. (1998). Herbivore-infested Plants Selectively Attract Parasitoids. Nature. 393, 570-573.

de S Pereira K, Guedes NMP, Serrão JE, Zanuncio JC, Guedes RNC. Superparasitism, immune response and optimum progeny yield in the gregarious parasitoid *Palmistichus elaeisis*. Pest Manag Sci. 2017; 73(6): 1101-1109. doi: 10.1002/ps.4534.

Del Mármol, J., Yedlin, M.A., and Ruta, V. (2021). The structural basis of odorant recognition in insect olfactory receptors. Nature. 597, 126-131.

Denis D, Pierre JS, van Baaren J, van Alphen JJ. Physiological adaptations to climate change in pro-ovigenic parasitoids. J Theor Biol. 2012; 309: 67-77. doi: 10.1016/j.jtbi.2012.06.005.

Diamandi, J.A., Duckhorn, J.C., Miller, K.E., Weinstock, M., Leone, S., Murphy, M.R., and Shirangi, T.R. (2024). Developmental remodeling repurposes larval neurons for sexual behaviors in adult *Drosophila*. Curr Biol. 34, 1183-1193.e3.

Dixson A. Sexual selection by cryptic female choice and the evolution of primate sexuality. Evol Anthropol. 2002; 11: 195-199.

Donaldson JS, Walter GH. Sex ratios of *Spalangia endius* (Hymenoptera: Pteromalidae), in relation to current theory. Ecol Entomol. 1984; 9: 395-402.

Dong, W.X., Zhang, F., Fang, Y.L., and Zhang, Z.N. (2008). Electroantennogram Responses of Aphid Parasitoid *Aphidius gifuensis* to Aphid Pheromones and Host-Plant Volatiles. Chin. J. Ecol. 27, 591-595.

Dorémus T, Jouan V, Urbach S, Cousserans F, Wincker P, Ravallec M, Wajnberg E, Volkoff AN. *Hyposoter didymator* uses a combination of passive and active strategies to escape from the *Spodoptera frugiperda* cellular immune response. J Insect Physiol. 2013; 59(4): 500-508. doi: 10.1016/j.jinsphys.2013.02.010.

Doucet D, Béliveau C, Dowling A, et al. Prophenoloxidases 1 and 2 from the spruce budworm, *Choristoneura fumiferana*: molecular cloning and assessment of transcriptional regulation by a polydnavirus. Arch Insect Biochem Physiol. 2010; 67(4): 188-201.

Duthie AB, Reid JM, Sander DG. Evolution of inbreeding avoidance and inbreeding preference through mate choice among interacting relatives. Am Nat. 2016; 188(6): 651-667. doi: 10.1086/688919.

E E Taylor, B H King, Edwin R Burgess. Diet and Nutrition of Adult *Spalangia cameroni* (Hymenoptera: Pteromalidae), a Parasitoid of Filth Flies. Environ Entomol. 2022; 51(1): 32-43.

Eijs IEM, Ellers J, Van Duinen GJ. Feeding strategies in drosophilid parasitoids: the impact of natural food resources on energy reserves in females. Ecol Entomol. 2010; 23(2): 133-138. doi: 10.1046/j.1365-2311.1998.00117.x.

Elias J, Mazzi D, Dorn S. No need to discriminate? Reproductive diploid males in a parasitoid with complementary sex determination. PLoS One. 2009; 4(6): e6024.

Ellers J, Ruhe B, Visser B. Discriminating between energetic content and. Entomol Exp Appl. 2011.

Elzinga JA, Zwakhals K, Harvey JA, Biere A. The parasitoid complex associated with the herbivore *Hadena bicruris* (Lepidoptera: Noctuidae) on *Silene latifolia* (Caryophyllaceae) in the Netherlands. J Nat Hist. 2007; 41: 101-123.

Emms DM, Kelly S. OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biol. 2019;20(1):238. https://doi.org/10.1186/s13059-019-1832-y

Eslin P, Giordanengo P, Fourdrain Y, et al. Avoidance of encapsulation in the absence of VLP by a braconid parasitoid of *Drosophila* larvae: an ultrastructural study. Can J Zool. 1996; 74(12): 2193-2198.

F. Wäckers. Assessing the suitability of flowering herbs as parasitoid food sources: flower attractiveness and nectar accessibility. Biol Control. 2004; 29: 307-314.

Fang Q, Wang BB, Ye XH, Wang F, Ye GY. Venom of parasitoid *Pteromalus puparum* impairs host humoral antimicrobial activity by decreasing host cecropin and lysozyme gene expression. Toxins. 2016; 8(2): 52. doi: 10.3390/toxins8020052.

Fatouros NE, Van Loon JJA, Hordijk KA, et al. Herbivore-induced plant volatiles mediate in-flight host discrimination by parasitoids. J Chem Ecol. 2005. doi: 10.1007/s10886-005-6076-5.

Fedina TY. Cryptic female choice during spermatophore transfer in *Tribolium castaneum* (Coleoptera: Tenebrionidae). J Insect Physiol. 2007; 53(1): 93-98. doi: 10.1016/j.jinsphys.2006.10.011.

Flynn JM, Hubley R, Goubert C, et al. RepeatModeler2 for automated genomic discovery of transposable element families. Proc Natl Acad Sci USA 2020;117(17):9451-9457. https://doi.org/10.1073/pnas.1921046117

Foti MC, Rostás M, Peri E, Park KC, Slimani T, Wratten SD, Colazza S. Chemical ecology meets conservation biological control: identifying plant volatiles as predictors of floral resource suitability for an egg parasitoid of stink bugs. J Pest Sci. 2017; 90: 299-310. doi: 10.1007/s10340-016-0758-3.

G. Benelli, G. Giunti, A. Tena, N. Desneux, A. Caselli, A. Canale. The impact of adult diet on parasitoid reproductive performance. J Pest Sci. 2017; 90(3): 807-823.

G. Siekmann, B. Tenhumberg, MA Keller. Feeding and survival in parasitic wasps: sugar concentration and timing matter. Oikos. 2001; 95: 425-430.

Gallot A, Sauzet S, Desouhant E. Kin recognition: Neurogenomic response to mate choice and sib mating avoidance in a parasitic wasp. PLoS One. 2020; 15(10): e0241128. doi: 10.1371/journal.pone.0241128.

Gandon S, Rivero A, Varaldi J. Superparasitism Evolution: Adaptation or Manipulation? Am Nat. 2006; 167: E1-E22. doi: 10.1086/498398.

Gao HS, Hu RM, Wang ZH, Ye XQ, Wu XT, Huang JH, Wang ZZ, Chen XX. A polydnavirus protein tyrosine phosphatase negatively regulates the host phenoloxidase pathway. Viruses. 2022; 15(1): 56. doi: 10.3390/v15010056.

Gardner A, Hardy ICW. Adjustment of sex allocation to co-foundress number and kinship under local mate competition: An inclusive-fitness analysis. J Evol Biol. 2020; 33(12): 1806-1812. doi: 10.1111/jeb.13719.

Gauld ID, Bolton B. The Hymenoptera. Oxford Univ. Press/The Nat Hist Mus London, Oxford. 1988.

Gerling, D. (1983). Observations of the biologies and interrelationships of parasites attacking the greenhouse whitefly, *Trialeurodes vaporariorum* (West.), in Hawaii. Proceedings of the Hawaiian Entomological Society. 24, 217-225.

Gilbert FS, Jervis MA. Functional, evolutionary, and ecological aspects of feeding-related mouthpart specializations in parasitoid flies. Biol J Linn Soc. 1998; 63: 495-535.

Godfray HCJ, Hunter MS. (1992). Sex ratios of heteronomous hyperparasitoids: adaptive or non-adaptive?. *Ecological Entomology*, *17*(1), 89-90. https://doi.org/10.1111/j.1365-2311.1992.tb01045.x

Godfray HCJ, Hunter MS. (1994). Heteronomous hyperparasitoids, sex ratios and adaptations: a reply. *Ecological Entomology*, *19*(1), 93-95. https://doi.org/10.1111/j.1365-2311.1994.tb00397.x

Godfray HCJ, Waage JK. (1990). The Evolution of Highly Skewed Sex Ratios in Aphelinid Wasps. *The American Naturalist*, *136*(5), 715-721. https://doi.org/10.1086/285126

Godfray, H.C.J. (1994). Parasitoids: Behavioural and evolutionary ecology. Princeton University Press, New Jersey.

Goubault M, Outreman Y, Poinsot D, Cortesero AM. Patch exploitation strategies of parasitic wasps under intraspecific competition. Behav Ecol. 2005; 16: 693-701.

Goubault, M., Cortesero, A.M., Paty, C., Fourrier, J., Dourlot, S., and Le Ralec, A. (2011). Abdominal sensory equipment involved in external host discrimination in a solitary parasitoid wasp. Microsc Res Tech. 74, 1145-1153.

Greany, P.D., and Oatman, E.R. (1972) Analysis of host discrimination in the parasite *Orgilus lepidus* (Hymenoptera: Braconidae). Ann. Entomol. Soc. Am. 65, 377-383.

Grillenberger BK, Van de Zande L, Bijlsma R, Gadau J, Beukeboom LW. Reproductive strategies under multiparasitism in natural populations of the parasitoid wasp *Nasonia* (Hymenoptera). J Evol Biol. 2009; 22(3): 460-470. doi: 10.1111/j.1420-9101.2008.01677.x.

Gu HN, Wang Q, Dorn S. Superparasitism in *Cotesia glomerata*: response of hosts and consequences for parasitoids. Ecol Entomol. 2003; 28: 422-431.

Gueguen G, Rajwani R, Paddibhatla I, Morales J, Govind S. VLPs of *Leptopilina boulardi* share biogenesis and overall stellate morphology with VLPs of the heterotoma clade. Virus Res. 2011; 160(1-2): 159-165. doi: 10.1016/j.virusres.2011.06.005.

Guillot, F.S., Joiner, R.L., and Vinson, S.B. (1974). Host discrimination: isolation of Hydrocarbons from Dufour's gland of a braconid parasitoid. Ann. Entomol. Soc. Am. 67, 720-721.

Gulinuer A, Xing B, Yang L. Host transcriptome analysis of *Spodoptera frugiperda* larvae parasitized by *Microplitis manilae*. Insects. 2023; 14(2): 100. doi: 10.3390/insects14020100.

Guo X, Kang L. Phenotypic plasticity in locusts: trade-off between migration and reproduction. Annu Rev Entomol. 2024. doi: 10.1146/annurev-ento-013124-124333.

Gurr GM, Liu J, Pogrebna G. Harnessing artificial intelligence for analysing the impacts of nectar and pollen feeding in conservation biological control. Curr Opin Insect Sci. 2024; 62: 101176. doi: 10.1016/j.cois.2024.101176.

Haas BJ, Salzberg SL, Zhu W, et al. Automated eukaryotic gene structure annotation using EVidenceModeler and the program to assemble spliced alignments. Genome Biol. 2008;9(1). https://doi.org/10.1186/gb-2008-9-1-r7

Haccou P, Glaizot O, Cannings C. Patch leaving strategies and superparasitism: an asymmetric generalized war of attrition. J Theor Biol. 2003; 225(1): 77-89. doi: 10.1016/s0022-5193(03)00223-6.

Hamelin F, Bernhard P, Nain P, Wajnberg E. Foraging under competition: evolutionarily stable patch-leaving strategies with random arrival times. In: Jørgensen S, Quincampoix M, Vincent T, eds. Advances in dynamic game theory. Birkhauser, Basel. 2007:349-365.

Hamilton WD. Extraordinary sex ratios. Science. 1967; 156: 477-480.

Han LB, Huang LQ, Wang CZ. Host preference and suitability in the endoparasitoid *Campoletis chlorideae* is associated with its ability to suppress host immune responses. Ecol Entomol. 2013; 38(2): 173-182.

Hardy ICW, Griffiths NT, Godfray HCJ. Clutch size in a parasitoid wasp: a manipulation experiment. J Anim Ecol. 1992; 61: 121-129.

Harvey JA, Cloutier J, Visser B, Ellers J, Wäckers FL, Gols R. The effect of different dietary sugars and honey on longevity and fecundity in two hyperparasitoid wasps. J Insect Physiol. 2012; 58(6): 816-823. doi: 10.1016/j.jinsphys.2012.03.002.

Harvey JA, Essens TA, Las RA, van Veen C, Visser B, Ellers J, Heinen R, Gols R. Honey and honey-based sugars partially affect reproductive trade-offs in

parasitoids exhibiting different life-history and reproductive strategies. J Insect Physiol. 2017; 98: 134-140. doi: 10.1016/j.jinsphys.2016.12.003.

Harvey JA, Gols R, Strand MR. Intrinsic competition and its effects on the survival and development of three species of endoparasitoid wasps. Entomol Exp Appl. 2009; 130(3): 238-248. doi: 10.1111/j.1570-7458.2008.00816.x.

Harvey JA, Poelman EH, Tanaka T. (2013). Intrinsic Inter- and Intraspecific Competition in Parasitoid Wasps. *Annual Review of Entomology*, *58*(1), 333-351. https://doi.org/10.1146/annurev-ento-120811-153622

Harvey JA. Factors affecting the evolution of development strategies in parasitoid wasps: the importance of functional constraints and incorporating complexity. Entomol Exp Appl. 2005; 117: 1-13.

He YQ, Zhang YQ, Chen JN, Chen WL, Zeng XY, Chen HT, Ding W. Effects of *Aphidius gifuensis* on the feeding behavior and potato virus Y transmission ability of *Myzus persicae*. Insect Sci. 2018; 25(6): 1025-1034. doi: 10.1111/1744-7917.12476.

He, M., Chen, H., Yang, X., Gao, Y., Lu, Y., and Cheng, D. (2022). Gut bacteria induce oviposition preference through ovipositor recognition in fruit fly. Commun Biol. 5, 973.

Heimpel GE, de Boer JG. Sex determination in the hymenoptera. Annu Rev Entomol. 2008; 53: 209-230. doi: 10.1146/annurev.ento.53.103106.093433.

Hofstetter RW, Raffa KF, Halevy M. Oviposition behavior of the quasi-gregarious parasitoid, *Ooencyrtus kuvanae* (Hymenoptera: Encyrtidae). J Insect Sci. 2024; 24(1): 18. doi: 10.1093/jisesa/ieae018.

Holmes LA, Nelson WA, Lougheed SC. Strong effects of food quality on host life history do not scale to impact parasitoid efficacy or life history. Sci Rep. 2023; 13(1): 3528. doi: 10.1038/s41598-023-30441-1.

Hosken DJ, Stockley P. Benefits of polyandry: a life history perspective. J Evol Biol. 2003; 33: 173-194. doi: 10.1007/978-1-4757-5190-1_4.

Hougardy E, Hogg BN. (2022). Factors Affecting Progeny Production and Sex Ratio of *Gryon aetherium* (Hymenoptera: Scelionidae), a Candidate Biological Control Agent for *Bagrada hilaris* (Hemiptera: Pentatomidae). *Insects*, *13*(11), 1010. https://doi.org/10.3390/insects13111010

Hu HY, Zhu XL, Chen ZZ, Niu LM, Fu YG. (2010). Sex allocation of parasitoid wasps. *Chinese Bulletin of Entomology*, *47*(06), 1081-1088.

Hu J, Zhu XX, Fu WJ. Passive evasion of encapsulation in *Macrocentrus cingulum* Brischke (Hymenoptera: Braconidae), a polyembryonic parasitoid of *Ostrinia furnacalis* Guenée (Lepidoptera: Pyralidae). J Insect Physiol. 2003; 49(4): 367-375. doi: 10.1016/S0022-1910(03)00050-8.

Hu W, Liu B, Xia S, Ma N, Wang P, Lu Y. Impact of nutritional supplements on the fitness of the parasitoid *Binodoxys communis* (Gahan). Insects. 2024; 15(4): 245. doi: 10.3390/insects15040245.

Huang Y, Yang N, Qin Y, An F, Li Z, Wan F. (2016) Enhanced stability in host-parasitoid interactions with autoparasitism and parasitoid migration. *Journal of Theoretical Biology*, 393, 43-50. https://doi.org/10.1016/j.jtbi.2015.12.032

Huang, Z.Y., Zhang, Y.J., Liu, J.Y., Yang, Z.D., Lu, W., and Zheng, X.L. (2018). Ultrastructure of Female Antennal Sensilla of an Endoparasitoid Wasp, *Quadrastichus mendeli* Kim & La Salle (Hymenoptera: Eulophidae: Tetrastichinae). Microsc Microanal. 24, 431-441.

Huchard ES, Schliehe DS, Kappeler PM, Kraus C. The inbreeding strategy of a solitary primate, *Microcebus murinus*. J Evol Biol. 2017; 13: 128-140. doi: 10.1111/jeb.12992.

Hunter MS, Godfray HCJ. (1995). Ecological Determinants of Sex Allocation in an Autoparasitoid Wasp. *Journal of Animal Ecology*, *64*(1), 95-106. https://doi.org/10.2307/5830

Hunter MS, Nur U, Werren JH. (1993) Origin of males by genome loss in an autoparasitoid wasp. *Heredity*, *70*(2), 162–171. https://nature.dosf.top/articles/hdy199325

Hunter MS, Woolley JB. (2001). Evolution and behavioral ecology of heteronomous aphelinid parasitoids. *Annual Review of Entomology*, *46*(1), 251-290. https://doi.org/10.1146/annurev.ento.46.1.251

Hunter MS. (1989). Sex allocation and egg distribution of an autoparasitoid, *Encarsia pergandiella* (Hymenoptera: Aphelinidae). *Ecological Entomology*, *14*(1), 57-67. https://doi.org/10.1111/j.1365-2311.1989.tb00754.x

Hunter MS. (1993). Sex allocation in a field population of an autoparasitoid. *Oecologia*, *93*(3), 421-428. https://doi.org/10.1007/BF00317887

Ivens AB, Shuker DM, Beukeboom LW, Pen I. Host acceptance and sex allocation of *Nasonia* wasps in response to conspecifics and heterospecifics. Proc Biol Sci. 2009; 276(1673): 3663-3669. doi: 10.1098/rspb.2009.0977.

Jervis MA, Moe A, Heimpel GE. The evolution of parasitoid fecundity: a paradigm under scrutiny. Ecol Lett. 2012; 15(4): 357-364. doi: 10.1111/j.1461-0248.2012.01745.x.

Jiang K, Zhou Y, Cui W, Han YW, Chen P, Liao GM, Hou YM, Tang BZ. Histone acetylation enhancing host melanization in response to parasitism by an endoparasitoid wasp. Insects. 2024; 15(3): 161. doi: 10.3390/insects15030161.

Jiang, X., Qin, Y., Jiang, J., Xu, Y., Francis, F., Fan, J., and Chen, J. (2022) Spatial Expression Analysis of Odorant Binding Proteins in Both Sexes of the Aphid Parasitoid *Aphidius gifuensis* and Their Ligand Binding Properties. Front Physiol. 13, 877133.

Jimenez-Perez A, Wang Q. Sexual selection in *Cnephasia jactatana* (Lepidoptera: Tortricidae) in relation to age, virginity, and body size. Ann Entomol Soc Am. 2004; 97: 819-824.

Jones IM, Koptur S, von Wettberg EJ, Diamond S. The use of extrafloral nectar in pest management: overcoming context dependence. J Appl Ecol. 2017; 54(2): 489-499.

Jones P, Binns D, Chang HY, et al. InterProScan 5: genome-scale protein function classification. Bioinformatics. 2014;30(9):1236-40. https://doi.org/10.1093/bioinformatics/btu031

Kafle BD, Morawo T, Fadamiro H. Host-induced plant volatiles mediate ability of the parasitoid *Microplitis croceipes* to discriminate between unparasitized and parasitized *Heliothis virescens* larvae and avoid superparasitism. J Chem Ecol. 2020; 46(10): 967-977. doi: 10.1007/s10886-020-01218-x.

Kalvari I, Nawrocki EP, Ontiveros-Palacios N, et al. Rfam 14: expanded coverage of metagenomic, viral and microRNA families. Nucleic Acids Res. 2021;49(D1). https://doi.org/10.1093/nar/gkaa1047

Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000;28(1):27-30. https://doi.org/10.1093/nar/28.1.27

Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013;30(4):772-80. https://doi.org/10.1093/molbev/mst010

Katono K, Macfadyen S, Omongo CA, et al. Effect of *Bemisia tabaci* SSA1 host density and cassava genotype on host feeding capacity and parasitism by two Hymenoptera parasitoid species. Biocontrol Sci Technol. 2022;33(1):19–34. https://doi.org/10.1080/09583157.2022.2151976

Kaya S, Uçkan F, Er A. Immunosuppressive influence of parasitoid wasp *Pimpla turionellae* calyx fluid on host *Galleria mellonella* cell-mediated immune response and hemocyte viability. Bull Entomol Res. 2021; 111(6): 795-805. doi: 10.1017/S0007485321000924.

Keil, T. (1999). Morphology and development of the peripheral olfactory organs. In Hansson B (ed.), *Insect Olfaction*. New York: Springer-Verlag. 5-47.

Kidane, D., Ferrante, M., Man, X.M., Liu, W.X., Wan, F.H., and Yang, N.W. (2020). Cold Storage Effects on Fitness of the Whitefly Parasitoids *Encarsia sophia* and *Eretmocerus hayati*. Insects. 11, 428.

Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods. 2015;12(4):357-60. https://doi.org/10.1038/nmeth.3317

King BH, Lee HE. Test of the adaptiveness of sex ratio manipulation in a parasitoid wasp. Behav Ecol Sociobiol. 1994; 35: 437-443.

King BH. Mate location and the onset of sexual responsiveness in the parasitoid Wasp *Spalangia endius* (Hymenoptera: Pteromalidae). Environ Entomol. 2010; 35(5): 1390-1395.

Kingsford C. A fast, lock-free approach for efficient parallel counting of occurrences of k-mers. Bioinformatics. 2011;27(6):764-70. https://doi.org/10.1093/bioinformatics/btr011

Koren S, Walenz BP, Berlin K, et al. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. Genome Res. 2017;27(5):722-736. https://doi.org/10.1101/gr.215087.116

Korf I. Gene finding in novel genomes. BMC Bioinformatics 2004;14(5):59. https://doi.org/10.1186/1471-2105-5-59

Kouamé, K.L., and Mackauer, M. (1991). Influence of aphid size, age and behaviour on host choice by the parasitoid wasp *Ephedrus californicus*: A test of host-size models. Oecologia. 88, 197-203.

Kroiss J, Schmitt T, Strohm E. A selfish function of a parasitoid's symbiotic virus: suppression of the hosts' immune system facilitates hyperparasitism. Evolution. 2010; 64(12): 3523-3530.

Krombein KV, Hurd PD, Smith DR, Burks BD. Catalog of Hymenoptera in America North of Mexico. Smithsonian Institution Press. 1979.

Krüger AP, Garcez AM, Scheunemann T, Bernardi D, Nava DE, Garcia FRM. Reproductive biology of *Trichopria anastrephae* (Hymenoptera: Diapriidae), a biological control agent of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae). Neotrop Entomol. 2024; 53: 32-40. doi: 10.1007/s13744-023-01009-9.

Kuenzel NT. (1975). A differentialdifference equation model of population dynamics in a natural life system of *Trialrurodes packardi* (Homoptera: Aleyrodidae) on lmpatienu pallida. Ph.D. thesis, Cornell University, Ithaca, New York. https://www.elibrary.ru/item.asp?id=7158975

Kuske S, Kremer N, Purcell J, Mead EA, Lee AY, Paroi CV, Kelly N, Hurst GDD. The role of symbionts in shaping host immunity: Current understanding and future directions. Insects. 2024; 15(2): 90. doi: 10.3390/insects15020090.

Lane NJ, Swales LS. Immunocytological localization of polydnavirus and associated virus-like particles in parasitoid wasps: an ultrastructural study. Tissue Cell. 1985; 17(4): 583-598.

Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357-9. https://doi.org/10.1038/nmeth.1923

Larocca, A., Fanti, P., Romano, V.A, Marsicovetere, E., Isidoro, N., Romani, R., Ruschioni, S., Pennacchio, F., and Battaglia, D. (2007). Functional bases of host-acceptance behaviour in the aphid parasitoid *Aphidius ervi*. Physiol. Entomol. 32, 305-312.

Lavine MD, Beckage NE. Polydnaviruses: potent mediators of host immune suppression. J Insect Physiol. 1995; 41(6): 473-482.

Lavine MD, Beckage NE. Temporal pattern of parasitism-induced immunosuppression and host haemolymph polypeptide changes after parasitization of *Manduca sexta* larvae by *Cotesia congregata*. Insect Biochem Mol Biol. 1996; 26(2): 621-630.

Le Ralec, A., Rabasse, J.M., and Wajnberg, E. (1996). Comparative morphology of the ovipositor of some parasitic Hymenoptera in relation to characteristics of their hosts. Can. Entomol. 128, 413-433.

Lehtonen J, Malabusini S, Guo X, Hardy ICW. Individual- and group-level sex ratios under local mate competition: consequences of infanticide and reproductive dominance. Evol Lett. 2023; 7(1): 13-23. doi: 10.1093/evlett/qrac005.

Leonard JE, Boake CRB. Site-dependent aggression and mating behavior in three species of *Nasonia* (Hymenoptera: Pteromalidae). Anim Behav. 2006; 71: 641-647.

Lepeco A, Melo GAR. Synopsis of the wasp genus *Clystopsenella* Kieffer (Hymenoptera: Scolebythidae). Zootaxa. 2022; 5134(1): 125-134. doi: 10.11646/zootaxa.5134.1.6.

Li B, Du Z, Tian L, et al. Chromosome-level genome assembly of the aphid parasitoid *Aphidius gifuensis* using Oxford Nanopore sequencing and Hi-C technology. Mol Ecol Resour. 2021;21(3):941-954. https://doi.org/10.1111/1755-0998.13308

Li D, Brough B, Rees JW, Coste CFD, Yuan C, Fowler MS, Sait SM. Humidity modifies species-specific and age-dependent heat stress effects in an insect host-parasitoid interaction. Ecol Evol. 2024; 14(7): e70047. doi: 10.1002/ece3.70047.

Li HA statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics. 2011;27(21):2987-93. https://doi.org/10.1093/bioinformatics/btr509

Li ZX, Ji MQ, Zhang C, Yang YB, Chen ZZ, Zhao HP, Xu YY, Kang ZW. The influence of host aphids on the performance of *Aphelinus asychis*. Insects. 2022; 13(9): 795. doi: 10.3390/insects13090795.

Li, R.T., Huang, L.Q., Dong, J.F., and Wang, C.Z. (2020). A moth odorant receptor highly expressed in the ovipositor is involved in detecting host-plant volatiles. Elife. 21, e53706.

Liu X, Zhang Y, Xie W, Wu Q, Wang S. The suitability of biotypes Q and B of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) at different nymphal instars as hosts for *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae). PeerJ. 2016; 4: e1863. doi: 10.7717/peerj.1863.

Lucie SM, Desneux N, Heimpel GE. Parasitoid-mediated indirect interactions between unsuitable and suitable hosts generate apparent predation in microcosm and modeling studies. Ecol Evol. 2021; 11(6): 2449-2460. doi: 10.1002/ece3.6896.

Lv, D., Zhao, H., Zhao, X.P., Zhao, M., Zhang, H.B., Zhao, G.S., Chen, M., and Wang, G.Y. (2020). Ultrastructural observation of female adults antennae, maxipalp and ovipositor sensilla of *Megastigmus sabinae* Xu et He. Environ. Entomol. 42, 1028-1036.

Mackauer M, Chau A. Adaptive self-superparasitism in a solitary parasitoid wasp: the influence of clutch size and offspring size. Funct Ecol. 2001; 15: 335-343.

Majoros WH, Pertea M, Salzberg SL. TigrScan and GlimmerHMM: two open-source ab initio eukaryotic gene-finders. Bioinformatics. 2004;20(16):2878-9. https://doi.org/10.1093/bioinformatics/bth315

Man XM, Sun LY, Frédéric F, Yang NW, Liu WX. Benefits of remating of a hyperparasitoid acting as a biocontrol agent. Biol Control. 2024; 197: 104996.

Man XM, Sun LY, Frédéric F, Yang NW, Liu WX. Can heteronomous hyperparasitoids recognize host abundance and adjust offspring ratio? Entomol Gen. 2024; DOI: 10.1127/entomologia/2024/2508.

Manni M, Berkeley MR, Seppey M, et al. BUSCO Update: Novel and streamlined workflows along with broader and deeper phylogenetic coverage for scoring of Eukaryotic, Prokaryotic, and Viral genomes. Mol Biol Evol. 2021;38(10):4647-4654. https://doi.org/10.1093/molbev/msab199

Martel V, Damiens D, Boivin G. Strategic ejaculation in the egg parasitoid *Trichogramma turkestanica* (Hymenoptera: Trichogrammatidae). Ecol Entomol. 2008; 33: 357-361.

Martínez-Martínez L, Leyva-Vázquez JL, Mojica HB. Sperm utilization in females of *Diachasmimorpha longicordata*. Southwestern Entomologist. 1993; 18: 293-299.

Mei Y, Jing D, Tang S, et al. InsectBase 2.0: a comprehensive gene resource for insects. Nucleic Acids Res. 2022;50(D1). https://doi.org/10.1093/nar/gkab1090

Mendel Z. Hymenopterous parasitoids of bark beetles (Scolytidae) in Israel: relationships between host and parasitoid size and sex ratio. Entomophaga. 1986; 31: 127-137.

Meng X, Ji Y. Modern Computational Techniques for the HMMER Sequence Analysis. ISRN Bioinform. 2013;2013:252183. https://doi.org/10.1155/2013/252183

Metzger M, Bernstein C, Hoffmeister TS, Desouhant E. Does kin recognition and sib-mating avoidance limit the risk of genetic incompatibility in a parasitic wasp? PLoS One. 2010; 5(10): e13505. doi: 10.1371/journal.pone.0013505.

Michaud, J.P., and Mackauer, M. (1994). The use of visual cues in host evaluation by aphidiid wasps. I. Comparison between three *Aphidius* parasitoids of the pea aphid. Entomol. Exp. Appl. 70, 273-283.

Michaud, J.P., and Mackauer, M. (1995). The use of visual cues in host evaluation by aphid wasps. II. Comparison between *Ephedrus californicus*, *Monoctonus paulensis*, and *Praon pequodorum*. Entomol. Exp. Appl. 74, 267-275.

Mills NJ, Gutierrez A. Prospective modelling in biological control: An analysis of the dynamics of heteronomous hyperparasitism in a cotton-whitefly-parasitoid system. J Appl Ecol. 1996;33(6):1379-1394. https://doi.org/10.2307/2404778

Misof B, Liu S, Meusemann K, et al. Phylogenomics resolves the timing and pattern of insect evolution. Science 2014;346(6210):763-7. https://doi.org/10.1126/science.1257570

Mohamad R, Monge JP, Goubault M. Agonistic interactions and their implications for parasitoid species coexistence. Behav Ecol. 2011; 22: 1114-1122. doi: 10.1093/beheco/arr098.

Mohamad R, Monge JP, Goubault M. Wait or fight? Ownership asymmetry affects contest behaviors in a parasitoid wasp. Behav Ecol. 2012; 23: 1330-1337. doi: 10.1093/beheco/ars125.

Mohamad R, Wajnberg E, Monge JP, Goubault M. The effect of direct interspecific competition on patch exploitation strategies in parasitoid wasps. Oecologia. 2015; 177(1): 305-315. doi: 10.1007/s00442-014-3124-2.

Moiroux J, Boivin G, Brodeur J. Ovigeny index increases with temperature in an aphid parasitoid: is early reproduction better when it is hot? J Insect Physiol. 2018; 109: 157-162. doi: 10.1016/j.jinsphys.2018.06.001.

Montoya, P., Benrey, B., Barrera, J.F., Zenil, M., Ruiz, L., and Liedo, P. (2003) Oviposition behavior and conspecific host discrimination in *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae), a fruit fly parasitoid. Biocontrol Sci Technol. 13, 683-690.

Morales J, Chiu H, Oo T, Plaza R, Hoskins S, Govind S. Biogenesis, structure, and immune-suppressive effects of virus-like particles of a *Drosophila* parasitoid, *Leptopilina victoriae*. J Insect Physiol. 2005; 51(2): 181-195. doi: 10.1016/j.jinsphys.2004.11.002.

Nakamatsu Y, Tanaka T. Development of a gregarious ectoparasitoid, *Euplectrus separatae* (Hymenoptera: Eulophidae), that parasitizes *Pseudaletia separata* (Lepidoptera: Noctuidae). Arthropod Struct Dev. 2003; 32(4): 329-336. doi: 10.1016/j.asd.2003.08.001.

Nakashima, Y., Birkett, M.A., Pye, B.J., and Powell, W. (2006). Chemically mediated intraguild predator avoidance by aphid parasitoids: interspecific variability in sensitivity to semiochemical trails of ladybird predators. J Chem Ecol. 32, 1989-1998.

Nawrocki EP, Eddy SR. Infernal 1.1: 100-fold faster RNA homology searches. Bioinformatics. 2013;29(22):2933-5. https://doi.org/10.1093/bioinformatics/btt509

Nguyen LT, Schmidt HA, von Haeseler A, et al. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol. 2015;32(1):268-74. https://doi.org/10.1093/molbev/msu300

Nufio CR, Papaj DR. Host marking behavior in phytophagous insects and parasitoids. Entomol Exp Appl. 2001; 99: 273-283.

Obonyo, M., Schulthess, F., Le Ru, B., Van Den Berg, J., Silvain, J.F., and Calatayud, P.A. (2010b) Importance of contact chemical cues in host recognition and acceptance by the braconid larval endoparasitoids *Cotesia sesamiae* and *Cotesia flavipes*. Biol Control. 54, 270-275.

Obonyo, M., Schulthess, F., Le Ru, B., Van Den Berg. J., and Calatayud, P.A. (2010a) Host recognition and acceptance behaviour in *Cotesia sesamiae* and *Cotesia flavipes* (Hymenoptera: Braconidae), parasitoids of gramineous stemborers in Africa. Eur J Entomol. 107, 169-176.

Ochieng, S.A., Park, K.C., Zhu, J.W., and Baker, T.C. (2000). Functional morphology of antennal chemoreceptors of the parasitoid *Microplitis croceipes* (Hymenoptera: Braconidae). Arthropod Struct Dev. 29, 231-240.

Ode PJ, Antolin MF, Strand MR. Brood-mate avoidance in the parasitic wasp *Bracon hebetor* Say. Anim Behav. 1995; 49(5): 1239-1248. doi: 10.1016/j.anbehav.1995.0156.

Ode PJ, Heinz KM. (2002). Host-size-dependent sex ratio theory and improving mass-reared parasitoid sex ratios. *Biologal control*, *24*(1), 31-41. https://doi.org/10.1016/S1049-9644(02)00003-8

Oliver KM, Russell JA, Moran NA, Hunter MS. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc Natl Acad Sci U S A. 2003; 100(4): 1803-1807. doi: 10.1073/pnas.0335320100.

O'Loughlin BS, Marcondes RS. Digest: when sexual selection hits the wall of natural selection. Evolution. 2024; 78(7): 1347-1348. doi: 10.1093/evolut/qpae068.

Otto M, Mackauer M. The developmental strategy of an idiobiont ectoparasitoid, *Dendrocerus carpenteri*: influence of variations in host quality on offspring growth and fitness. Oecologia. 1998; 117(3): 353-364. doi: 10.1007/s004420050668.

Pang L, Fang G, Liu Z, Dong Z, Chen J, Feng T, Zhang Q, Sheng Y, Lu Y, Wang Y, Zhang Y, Li G, Chen X, Zhan S, Huang J. Coordinated molecular and ecological adaptations underlie a highly successful parasitoid. Elife. 2024; 13: RP94748. doi: 10.7554/eLife.94748.

Pang, X.N., Liu, X.H., Liu, G.T., Li, X.Y., Wang, Q.K., and Zhang, D. (2020). Morphology and functional implication of antennal sensilla in *Calyptratae*. Environ. Entomol. 42, 370-382.

Papp, J. (1974). A study on the systematics of Braconidae (Hymenoptera). Folia Entomolgica Hungarica. 27, 109-133.

Parra G, Bradnam K, Korf I. CEGMA: a pipeline to accurately annotate core genes in eukaryotic genomes. Bioinformatics. 2007;23(9):1061-7. https://doi.org/10.1093/bioinformatics/btm071

Pennacchio F, Caccia S, Digilio MC. Host regulation and nutritional exploitation by parasitic wasps. Curr Opin Insect Sci. 2014; 6: 74-79. doi: 10.1016/j.cois.2014.09.018.

Pertea M, Pertea GM, Antonescu CM, et al. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol. 2015;33(3):290–295. https://doi.org/10.1038/nbt.3122

Peters RS, Krogmann L, Mayer C, et al. Evolutionary history of the Hymenoptera. Curr Biol. 2017;27(7):1013-1018. https://doi.org/10.1016/j.cub.2017.01.027

Pinto CPG, Walker AA, King GF, Rossi GD. Immunosuppressive, antimicrobial and insecticidal activities of inhibitor cystine knot peptides produced by teratocytes of the endoparasitoid wasp *Cotesia flavipes* (Hymenoptera: Braconidae). Insect Sci. 2023; 30(4): 1105-1117. doi: 10.1111/1744-7917.13154.

Polaszek A, Vilhemsen L. Biodiversity of hymenopteran parasitoids. Curr Opin Insect Sci. 2023; 56: 101026. doi: 10.1016/j.cois.2023.101026.

Quicke DLJ. Parasitic Wasps. Chapman & Hall, London. 1997.

Quicke DLJ. Phylogeny and systematics of the Ichneumonidae. In: The Braconid and Ichneumonid Parasitoid Wasps: Biology, Systematics, Evolution and Ecology. Wiley and Sons, New York. 2015.

Ramadan MM, Wang X. Male impact on female reproductive performance of the larval tephritid parasitoid *Diachasmimorpha tryoni* (Hymenoptera: Braconidae). J Econ Entomol. 2021; 114(3): 1117-1127. doi: 10.1093/jee/toab063.

Ramirez-Villagas M, Ochoa-Rivera G, Hummel RL, Montoya P, Aluja M. Nonlinear dynamics of parasitism in an agriculturally important tritrophic system. J Anim Ecol. 2024; 93(5): 1010-1025.

Reineke A, Asgari S, Schmidt O. Evolutionary origin of *Venturia canescens* virus-like particles. Arch Insect Biochem Physiol. 2006; 61(3): 123-133. doi: 10.1002/arch.20113.

Rhie A, Walenz BP, Koren S, et al. Merqury: reference-free quality, completeness, and phasing assessment for genome assemblies. Genome Biol. 2020;21(1):245. https://doi.org/10.1186/s13059-020-02134-9

Riccardo F, Jacob R, Vincenzo G, Isabel MS, Luca M. (2018). Host instar influence on offspring sex ratio and female preference of *Neodryinus typhlocybae* (Ashmead) (Hymenoptera, Dryinidae) parasitoid of *Metcalfa pruinosa* (Say)

(Homoptera, Flatidae). *Biological Control*, 125, 113-120. https://doi.org/10.1016/j.biocontrol.2018.05.009

Richards EH, Edwards JP. Larvae of the ectoparasitic wasp, *Eulophus pennicornis*, release factors which adversely affect haemocytes of their host, *Lacanobia oleracea*. J Insect Physiol. 2002; 48(9): 845-855. doi: 10.1016/s0022-1910(02)00154-3.

Richards EH. Salivary secretions from the ectoparasitic wasp, *Eulophus pennicornis* contain hydrolases, and kill host hemocytes by apoptosis. Arch Insect Biochem Physiol. 2012; 79(2): 61-74. doi: 10.1002/arch.21006.

Rivero A, West SA. The physiological costs of being small in a parasitic wasp. Evol Ecol Res. 2002; 4(3): 407-420.

Roberts HLS, Schmidt O. Lifetime egg maturation by host-deprived *Venturia canescens*. J Insect Physiol. 2004; 50: 195-202.

Rosenheim JA, Heimpel GE, Mangel M. Egg maturation, egg resorption and the costliness of transient egg limitation in insects. Proc Biol Sci. 2000; 267(1452): 1565-1573. doi: 10.1098/rspb.2000.1179.

Rosi, M.C., Isidoro, N.S., and Colazza, F.B. (2001). Source of the host marking pheromone in the egg parasitoid *Trissolcus basalis* (Hymenoptera: Scelionidae). J. Insect Physiol. 47, 989-995.

Roy S, Saha TT, Zou Z, Raikhel AS. Regulatory pathways controlling female insect reproduction. Annu Rev Entomol. 2018; 63: 489-511. doi: 10.1146/annurev-ento-020117-043258.

Salvia R, Grimaldi A, Girardello R, Scieuzo C, Scala A, Bufo SA, Vogel H, Falabella P. *Aphidius ervi* teratocytes release enolase and fatty acid binding protein through exosomal vesicles. Front Physiol. 2019; 10: 715. doi: 10.3389/fphys.2019.00715.

Salvia R, Scieuzo C, Boschi A, Pezzi M, Mistri M, Munari C, Chicca M, Vogel H, Cozzolino F, Monaco V, Monti M, Falabella P. An overview of ovarian calyx fluid proteins of *Toxoneuron nigriceps* (Viereck) (Hymenoptera: Braconidae): An integrated transcriptomic and proteomic approach. Biomolecules. 2023; 13(10): 1547. doi: 10.3390/biom13101547.

Sandlan KP. Host suitability and its effects on parasitoid biology in *Coccygomimus turionellae* (Hymenoptera: Ichneumonidae). Ann Entomol Soc Am. 1982; 75: 217-221.

Sarikaya A, Gülel A. Effects of host species, stage and size on the sex ratio and clutch size of the parasitoid, *Dibrachys boarmiae* (Walker, 1863) (Hymenoptera: Pteromalidae). Bull Entomol Res. 2011; 101(3): 325-331. doi: 10.1017/S0007485310000532.

Saunders TE, Manning LM, Avila GA, Holwell GI, Park KC. Electrophysiological responses of *Trissolcus japonicus*, *T. basalis*, and *T. oenone* (Hymenoptera: Scelionidae) to volatile compounds associated with New Zealand stink bugs (Hemiptera: Pentatomidae). J Chem Ecol. 2024; doi: 10.1007/s10886-024-01533-7.

Savino V, Luna MG, Salas Gervassio NG, Coviella CE. Interspecific interactions between two *Tuta absoluta* (Lepidoptera: Gelechiidae) larval parasitoids with contrasting life histories. Bull Entomol Res. 2017; 107(1): 32-38. doi: 10.1017/S0007485316000547.

Schmidt O, Li D, Beck M, et al. Phenoloxidase-like activities and the function of virus-like particles in ovaries of the parthenogenetic parasitoid *Venturia canescens*. J Insect Physiol. 2005; 51(2): 117-125.

Sequeria R, Mackauer M. Variation in selected life history parameters of the parasitoid wasp, *Aphidius ervi*: influence of host development stage. Entomol Exp Appl. 1994; 71: 15-22.

Serbielle C, Dupas S, Perdereau E, Héricourt F, Dupuy C, Huguet E, Drezen JM. Evolutionary mechanisms driving the evolution of a large polydnavirus gene family coding for protein tyrosine phosphatases. BMC Evol Biol. 2012; 12: 253. doi: 10.1186/1471-2148-12-253.

Shah, Z.A. (2012). Morphology, ultrastructure, and probable functions of the sense organs on the ovipositor stylets of the hymenoptran parasitoid, *Venturia canescens* (Gravenhorst). Microsc Res Tech. 75, 876-883.

Shahbazvar, N., Hosseini, R., and Hayat, M. (2022). A new species of *Coccobius* Ratzeburg (Hymenoptera: Chalcidoidea: Aphelinidae) from Iran, with new host records for two species. Zootaxa. 4, 588-592.

Shirota, Y., Carter, N., Rabbinge, R., and Ankersmit, G. (1983). Biology of *Aphidius rhopalosiphi*, a parasitoid of cereal aphids. Entomol. Exp. Appl. 34, 27-34.

Shuker DM, West SA. Information constraints and the precision of adaptation: sex ratio manipulation in wasps. Proc Natl Acad Sci U S A. 2004; 101: 10363-10367.

Simmons LW. The evolution of polyandry: an examination of the genetic incompatibility and good-sperm hypotheses. J Evol Biol. 2001; 14(4): 585-594.

Sisterson MS, Averill AL. Costs and benefits of food foraging for a braconid parasitoid. J Insect Behav. 2002; 15(4): 571-588. doi: 10.1023/A:1016389402543.

Slifer, E.H. (1970). The structure of arthropod chemoreceptors. Annu Rev Entomol. 15, 121-142.

Snodgrass, R.E. (1931). Morphology of the insect abdomen. Part I: General structure of the abdomen and its appendages. Smithsonian Institution Miscellaneous Collections, 85, 1-128.

Snodgrass, R.E. (1935). Morphology of the insect abdomen. Part II: The genital ducts and the ovipositor. Smithsonian Institution Miscellaneous Collections, 85, 1-148.

Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30(9):1312-3. https://doi.org/10.1093/bioinformatics/btu033

Stanke M, Keller O, Gunduz I, et al. AUGUSTUS: ab initio prediction of alternative transcripts. Nucleic Acids Res. 2006;34–439. https://doi.org/10.1093/nar/gkl200

Steinbrecht, R.A. (1997). Pore structures in insect olfactory sensilla: A review of data and concepts. Int J Morphol. 26, 229-245.

Strand MR. Teratocytes and their functions in parasitoids. Curr Opin Insect Sci. 2014; 6: 68-73. doi: 10.1016/j.cois.2014.09.005.

Straser RK, Daane KM, Stahl JM, Wilson H. Floral resources enhance fitness of the parasitoid *Hadronotus pennsylvanicus* (Hymenoptera: Scelionidae) but not biological control of its host *Leptoglossus zonatus* (Heteroptera: Coreidae). Environ Entomol. 2024; 53(2): 213-222. doi: 10.1093/ee/nvae002.

Sun. (2014). Reproductive characteristics and sex ratio regulation mechanism of *Encarsia sophia* (Girault & Dodd) (Hymenoptera: Aphelinidae). Southwest University.

Supek F, Bošnjak M, Škunca N, et al. REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS One. 2011;6(7). https://doi.org/10.1371/journal.pone.0021800

Tamò C, Roelfstra LL, Guillaume S, Turlings TC. Odour-mediated long-range avoidance of interspecific competition by a solitary endoparasitoid: a time-saving foraging strategy. J Anim Ecol. 2006; 75(5): 1091-1099. doi: 10.1111/j.1365-2656.2006.01128.x.

Tarailo-Graovac M, Chen N. Using RepeatMasker to identify repetitive elements in genomic sequences. Curr Protoc Bioinformatics 2009;4:4.10.1-4.10.14. https://doi.org/10.1002/0471250953.bi0410s25

Teng Z, Wu H, Ye X, Xiong S, Xu G, Wang F, Fang Q, Ye G. An ovarian protein involved in passive avoidance of an endoparasitoid to evade its host immune response. J Proteome Res. 2019; 18(7): 2695-2705. doi: 10.1021/acs.jproteome.8b00824.

Thornhill R, Alcock J. The evolution of insect mating systems. iUniverse.com, Inc., Lincoln. 1983.

Tize I, Nukenine EN, Fotso KA, Doumtsop FA, Nanga NS, Ajebesone FN, Kulakow P; Kumar PL, Fiaboe KKM, Hanna R. (2023). Parasitism of the whitefly bemisia tabaci by aphelinid parasitoids on cassava across five agro-ecological zones of cameroon. *Crop Protection*, 168. https://doi.org/10.1016/j.cropro.2023.106241.

Ueno T, Ueno K. The effects of host-feeding on synovigenic egg development in an endoparasitic wasp, *Itoplectis naranyae*. J Insect Sci. 2007; 7: 1-13. doi: 10.1673/031.007.4601.

Van Alphen JJM, Visser ME. Superparasitism as an adaptive strategy for insect parasitoids. Annu Rev Entomol. 1990; 35: 59-79. doi: 10.1146/annurev.en.35.010190.000423.

van Baaren J, Boivin G, Visser B, Le Lann C. Bet-hedging in parasitoids: when optimization is not the best strategy to cope with climatic extremes. Curr Res Insect Sci. 2024; 5: 100076. doi: 10.1016/j.cris.2024.100076.

Van Lenteren, J.C. (1981) Host discrimination by parasitoids. In Nordlund, AD, Jones, RL and Lewis, WL (eds), Semiochemicals: Their Role in Pest Control. New York: John Wiley and Sons, pp. 153-179.

van Lenteren, J.C., Ruschioni, S., Romani, R., van Loon, J.J., Qiu, Y.T., Smid, H.M., Isidoro, N., and Bin, F. (2007) Structure and electrophysiological responses of gustatory organs on the ovipositor of the parasitoid *Leptopilina heterotoma*. Arthropod Struct Dev. 36, 271-276.

Vet, L.E.M., and Dicke, M. (1992) Ecology of infochemical use by natural enemies in a tritrophic context. Annu Rev Entomol. 37, 141–172.

Vinson, S.B. (1975) Biochemical convolution between parasitoids and their hosts. In: Price PW (ed) Evolutionary strategies of parasitic insects and mites. Plenum Press, New York.

Vinson, S.B. (1976) Host selection by insect parasitoids. Annu Rev Entomol. 21, 109–134.

Vinson, S.B. (1985). The behaviour of parasitoids, pp. 417–469, in G.A. Kerkut and L.I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon, New York.

Waage JK, Lane JA. The reproductive strategy of a parasitic wasp. II. Sex allocation and local mate competition in *Trichogramma evanescens*. J Anim Ecol. 1983; 53: 417-426.

Wajnberg E, Coquillard P, Vet LE, Hoffmeister T. Optimal resource allocation to survival and reproduction in parasitic wasps foraging in fragmented habitats. PLoS One. 2012; 7(6): e38227. doi: 10.1371/journal.pone.0038227.

Wajnberg, E., and Colazza, S. (2013) Chemical ecology of insect parasitoids. Whiley-Blackwell, A John Wiley & Sons, Ltd., Publication, UK.

Wajnberg, E., Bernstein, C., and Van Alphen, J. (2008) Behavioral ecology of insect parasitoids. Blackwell Publishing, USA, UK, Australia.

Walter GH, Donaldson JS. (1994). Heteronomous hyperparasitoids, sex ratios and adaptations. *Ecological Entomology*, *19*(1), 89-92. https://doi.org/10.1111/j.1365-2311.1994.tb00396.x

Walter GH. Divergent male ontogenies in Aphelinidae (Hymenoptera, Chalcidoidea): A simplified classification and a suggested evolutionary sequence. Biol J Linn

Soc Lond. 1983;19(1):63-82. https://doi.org/10.1111/j.1095-8312.1983.tb00777.x

Wang W, Lu SL, Liu WX, Cheng LS, Zhang YB, Wan FH. Effects of five naturally occurring sugars on the longevity, oogenesis, and nutrient accumulation pattern in adult females of the synovigenic parasitoid *Neochrysocharis formosa* (Hymenoptera: Eulophidae). Neotrop Entomol. 2014; 43(6): 564-573. doi: 10.1007/s13744-014-0247-4.

Wang ZZ, Liu YQ, Shi M, Huang JH, Chen XX. Parasitoid wasps as effective biological control agents. J Integr Agric. 2019; 18(4): 705-715.

Wang, D., Lü, L., He, Y., Shi, Q., Tu, C., and Gu, J. (2016). Mate choice and host discrimination behavior of the parasitoid *Trichogramma chilonis*. Bull Entomol Res. 106, 530-7.

Warsi S, Chicas-Mosier AM, Balusu RR, Jacobson AL, Fadamiro HY. (2023). Effects of Food Source Availability, Host Egg:Parasitoid Ratios, and Host Exposure Times on the Developmental Biology of *Megacopta cribraria* Egg Parasitoids. *Insects*, *14*(9),755. https://doi.org/10.3390/insects14090755

Weseloh, R.M. (1972). Sense organs of the hyperparasite *Cheiloneurus noxius* (Hymenoptera: Encyrtidae) important in host selection processes. Ann. Entomol. Soc. Am. 65, 41-46.

West SA, Reece SE, Read AF. *Toxoplasma gondii*, sex and premature rejection. Trends Parasitol. 2003; 19(4): 155-157.

Williams JR. (1977) Some features of sex-linked hyperparasitism in Aphelinidae (Hymenoptera). *Entomophaga*, *22*, 345-350. https://doi.org/10.1007/BF02373258

Williams T. Invasion and displacement of experimental populations of a conventional parasitoid by a heteronomous hyperparasitoid. Biocontrol Sci Technol. 1996;6(4):603-618. https://doi.org/10.1080/09583159631244

Wolf M, van Doorn GS, Leimar O, Weissing FJ. Life-history tradeoffs favour the evolution of animal personalities. Nature. 2007; 447: 581-584. doi: 10.1038/nature05835.

Wu N, Zhang S, Li X, et al. Fall webworm genomes yield insights into rapid adaptation of invasive species. Nat Ecol Evol. 2019;3(1):105-115. https://doi.org/10.1038/s41559-018-0746-5

Wu Z, Yuan R, Gu Q, Wu X, Gu L, Ye X, Zhou Y, Huang J, Wang Z, Chen X. Parasitoid serpins evolve novel functions to manipulate host homeostasis. Mol Biol Evol. 2023; 40(12): msad269. doi: 10.1093/molbev/msad269.

Wulff, J.P., Traverso, L.M., Latorre-Estivalis, J.M., Segura, D.F., and Lanzavecchia, S.B. (2024). Identification of candidate genes associated with host-seeking behavior in the parasitoid wasp *Diachasmimorpha longicaudata*. BMC Genom. 25, 147.

Xu HY, Yang NW, Chi H, Ren GD, Wan FH. (2018). Comparison of demographic fitness and biocontrol effectiveness of two parasitoids, *Encarsia sophia* and *Eretmocerus hayati* (Hymenoptera: Aphelinidae), against *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Pest Management Science*, *74*(9), 2116-2124. https://doi.org/10.1002/ps.4908

Xu HY, Yang NW, Wan FH. (2013). Competitive interactions between parasitoids provide new insight into host suppression. *PLoS ONE*, *8*(11), e82003. https://doi.org/10.1371/journal.pone.0082003

Xu Z, Wang H. LTR_FINDER: an efficient tool for the prediction of full-length LTR retrotransposons. Nucleic Acids Res. 2007;35:265-268. https://doi.org/10.1093/nar/gkm286

Xu, L., Jiang, H.B., Yu, J.L., Lei, Q., Pan, D., Chen, Y., Dong, B., Liu, Z., and Wang, J.J. (2024). An Odorant Receptor Expressed in Both Antennae and Ovipositors Regulates Benzothiazole-Induced Oviposition Behavior in *Bactrocera dorsalis*. J Agric Food Chem. 13, 6954-6963.

Yang NW, Ji LL, Lövei GL, Wan FH. (2012). Shifting preference between oviposition vs. host- feeding under changing host densities in two aphelinid parasitoids. *PLoS ONE*, *7*, e41189. https://doi.org/10.1371/journal.pone.0041189

Yang S, Dou W, Li M, Wang Z, Chen G, Zhang X. (2022). Flowering agricultural landscapes enhance parasitoid biological control to *Bemisia tabaci* on tomato in south China. *PLoS One*, *17*(8), e0272314. https://doi.org/10.1371/journal.pone.0272314

Yang X, Qu YL, Wu ZY, Lin Y, Ruan CC, Desneux N, Zang LS. Parasitism and suitability of fertilized and nonfertilized eggs of the rice striped stem borer, *Chilo suppressalis* (Lepidoptera: Crambidae), for *Trichogramma* parasitoids. J Econ Entomol. 2016; 109(4): 1524-1528. doi: 10.1093/jee/tov388.

Yang Y, Ye X, Dang C, et al. Genome of the pincer wasp *Gonatopus flavifemur* reveals unique venom evolution and a dual adaptation to parasitism and predation. BMC Biol. 2021;19(1):145. https://doi.org/10.1186/s12915-021-01081-6

Yang Z. PAML 4: phylogenetic analysis by maximum likelihood. Mol Biol Evol. 2007;24(8):1586-91. https://doi.org/10.1093/molbev/msm088

Ye H, Yan Z, Yang Y, et al. A chromosome-level genome assembly of the parasitoid wasp *Pteromalus puparum*. Mol Ecol Resour. 2020;20(5):1384-1402. https://doi.org/10.1111/1755-0998.13206

Yin C, Li M, Hu J, Lang K, Chen Q, Liu J, Guo D, He K, Dong Y, Luo J, Song Z, Walters JR, Zhang W, Li F, Chen X. The genomic features of parasitism, polyembryony and immune evasion in the endoparasitic wasp *Macrocentrus cingulum*. BMC Genomics. 2018; 19: 420. doi: 10.1186/s12864-018-4783-x.

Zhang LW, Hu HY, Zhang YZ. (2023). Two new species of Coccophagus Westwood (Hymenoptera: Aphelinidae) from China. *Zootaxa*, 5258(3), 342-350. https://doi.org/10.11646/zootaxa.5258.3.7

Zhang S, Shen S, Peng J, et al. Chromosome-level genome assembly of an important pine defoliator, *Dendrolimus punctatus* (Lepidoptera; Lasiocampidae). Mol Ecol Resour. 2020;20(4):1023-1037. https://doi.org/10.1111/1755-0998.13169

Zhang X, Zhang S, Zhao Q, et al. Assembly of allele-aware, chromosomal-scale autopolyploid genomes based on Hi-C data. Nat Plants. 2019;5(8):833-845. https://doi.org/10.1038/s41477-019-0487-8

Zhang YH, Xue JZ, Tariq T, Li TH, Qian HY, Cui WH, Tian H, Monticelli LS, Desneux N, Zang LS. Parasitism and suitability of *Trichogramma chilonis* on large eggs of two factitious hosts: *Samia cynthia ricini* and *Antheraea pernyi*. Insects. 2023; 15(1): 2. doi: 10.3390/insects15010002.

Zhang Z, Xiao J, Wu J, et al. ParaAT: a parallel tool for constructing multiple protein-coding DNA alignments. Biochem Biophys Res Commun. 2012;419(4):779-81. https://doi.org/10.1016/j.bbrc.2012.02.101

Zhang, L., Sun, H., Grosse-Wilde, E., Zhang, L., Hansson, B.S., Dweck, and H.K.M. (2023). Cross-generation pheromonal communication drives *Drosophila* oviposition site choice. Curr Biol. 33, 2095-2103.e3.

Zhang, L.W., Hu, H.Y., and Zhang, Y.Z. (2023). Two new species of *Coccophagus* Westwood (Hymenoptera: Aphelinidae) from China. Zootaxa. 3, 342-350.

Zhang, X., Liu, Y., Guo, M., Sun, D., Zhang, M., Chu, X., Berg, B.G., and Wang, G. (2024). A female-specific odorant receptor mediates oviposition deterrence in the moth *Helicoverpa armigera*. Curr Biol. 34, 1-11.e4.

Zhang, Y., Tian, X., Wang, H., Castené, C., Arnó, J., Collatz, J., Romeis, J., Wu, S., Xian, X., Liu, W., Wan, F., and Zhang, G. (2021). Host selection behavior of the host-feeding parasitoid *Necremnus tutae* on *Tuta absoluta*. Entomol. Gen. 42, 445-456.

Zhao Y, Zhu L, Ramirez-Romero R, Dai P, Yang X, Ruan CC, Desneux N, Zang LS. (2022). Mating status of an autoparasitoid and sex of the secondary host impact the outcome of heteronomous hyperparasitism. *Entomologia generalis*. *42*(1), 87-99. https://doi.org/10.1127/entomologia/2021/1324

Zhao, Y., Zhu, L., Ramirez-Romero, R., Dai, P., Yang, X., Ruan, C.C, Desneux, N., and Zang, L.S. (2022). Mating status of an autoparasitoid and sex of the secondary host impact the outcome of heteronomous hyperparasitism. Entomol. Gen. 42, 87-99.

Zhong YW, Fan YY, Zuo ZQ, et al. A chromosome-level genome assembly of the parasitoid wasp *Eretmocerus hayati*. Sci Data. 2023;10(1):585. https://doi.org/10.1038/s41597-023-02450-2

Zhou L, Wang R, Lin Z, Shi S, Chen C, Jiang H, Zou Z, Lu Z. Two venom serpins from the parasitoid wasp *Microplitis mediator* inhibit the host prophenoloxidase activation and antimicrobial peptide synthesis. Insect Biochem Mol Biol. 2023; 152: 103895. doi: 10.1016/j.ibmb.2022.103895.

Zhu, X., Liu, S., Xu, C.Q., Guo, K., Xu, R., Qiao, H.L., and Chen, J. (2021). Advances in research on sensilla structure and function in parasitic wasps. Chinese Journal of Applied Entomology. 58, 822-845.

Appendices

List of publications

Accepted publications (peer reviewed)

1. **Man, X**., Sun, L., Francis, F., Yang, N., Liu, W. Benefits of remating of a hyperparasitoid acting as a biocontrol agent. *Biological Control*, 2024. 197, 105606 https://doi.org/10.1016/j.biocontrol.2024.105606

2. **Man, X**., Sun, L., Francis, F., Yang, N., Liu, W. Can heteronomous hyperparasitoids recognize host abundance and adjust offspring ratio? *Entomologia Generalis*, 2024. 44(4), 1017-1025 https://doi.org/10.1127/entomologia/2024/2508

3. **Man, X**., Huang, C., Wu, S., Guo, J., Wan, F., Francis, F., Yang, N., Liu, W. A chromosome-level genome assembly of the heteronomous hyperparasitoid wasp *Encarsia sophia*. *Scientific Data*. 11, 1250 (2024). https://doi.org/10.1038/s41597-024-04040-2

Prepare to submit articles

1. **Man, X**., Wu, S., Huang, C., Francis, F., Yang, N., Liu, W. Daughter or Son? Host Odor Determines Offspring Sex in Parasitoid. Submitting.

2. **Man, X**., Francis, F., Yang, N., Liu, W. The adaptive strategies of reproductive fitness in parasitoids wasps. Submitting.

Presentations at conferences (peer reviewed)

1. **Man, X**., Yang, N., Francis, F., Liu, W., Reproductive Benefits of Multiple Mating in *Encarsia sophia*. in 2023 *Conference on Biological Invasions*. Chongqing, China. Poster

2. **Man, X**., Yang, N., Liu, W., Francis, F., Oviposition decision-making mechanism of heteronomous hyperparasitoid *Encarsia sophia*. in *74th international symposium on crop protection (ISCP2024)*. 2024: Ghent, Belgium. Poster